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<b>(54) Title:</b> TRISTETRAPROLIN			
<b>(57) Abstract</b> <p>The present invention relates, in general, to tristetraprolin (TTP) and, in particular, to methods of modulating levels of tumor necrosis factor <math>\alpha</math> (TNF<math>\alpha</math>) using TTP or nucleic acid sequences encoding same. The invention further relates to methods of screening for compounds for their ability to inhibit TNF<math>\alpha</math> biosynthesis.</p>			

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**TRISTETRAPROLIN**

This is a continuation-in-part of Application No. 08/648,773, filed May 16, 1996, the entire contents of which are hereby incorporated by reference.

This application was made with Government support under Grant No. \_\_\_\_\_ awarded by the National Institutes of Health. The Government has certain rights in the invention.

**TECHNICAL FIELD**

The present invention relates, in general, to tristetraprolin (TTP) and, in particular, to methods of modulating levels of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) using TTP or nucleic acid sequences encoding same. The invention further relates to methods of screening compounds for their ability to inhibit TNF $\alpha$  biosynthesis, processing or secretion.

**BACKGROUND**

Tumor necrosis factor alpha (TNF $\alpha$ ) is a potent cytokine that is released from many cell types, particularly, macrophages and monocytes. TNF $\alpha$  also exists in a cell-membrane bound, higher molecular weight form on cells, and this form also appears to mediate a variety of biological effects. TNF $\alpha$  is thought to have few roles in normal development and physiology; however, it exerts harmful and destructive effects on many tissues in many disease states (Tracey et al, Ann. Rev. Med. 45:491 (1994)). Disease states in which TNF $\alpha$  has been shown to exert a major pathogenetic role include septic shock syndrome, cancer cachexia, rheumatoid arthritis, etc.

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Many investigators and pharmaceutical companies are actively investigating agents and potential drugs that can block TNF $\alpha$  effects, either by blocking its synthesis or interfering with its binding to its surface receptors.

One example of this approach is the use of monoclonal antibodies to TNF $\alpha$ . These have been used in animal models of human disease, and in human conditions such as rheumatoid arthritis (Arend et al, Arthritis and Rheumatism 38:151 (1995)). There is no question that these antibodies temporarily relieve some of the signs and symptoms of this disease in man. However, their potential widespread use is compromised by many factors, especially the fact that they seem to be only temporarily (ie, a few months) effective. Other drawbacks include expense, the need for parenteral administration, the likelihood that anti-idotype antibodies will develop, etc.

The present invention relates to a novel approach to the treatment of diseases the effects of which are mediated, at least in part, through TNF $\alpha$ . This approach involves the protein tristetraprolin (TTP) and nucleic acid sequences encoding same.

TTP (Lai et al, J. Biol. Chem. 265:16556 (1990)), also known as Nup475 (DuBois et al, J. Biol. Chem. 265:19185 (1990)) and TIS11 (Varnum et al, Oncogene 4:119 (1989); Varnum et al, Mol. Cell. Biol. 11:1754 (1991)), is a widely distributed 33.6 kDa phosphoprotein encoded by the immediate-early response gene, Zfp-36 (Taylor et al, Nucl. Acids Res. 19:3454 (1991)). This gene has been mapped to chromosome 7 in the mouse, and the equivalent human gene, ZFP36, has been mapped to chromosome 19q 13.1 (Taylor et al, Nucl. Acids Res. 19:3454 (1991)). TTP is the prototype of a group of proteins containing two or more highly conserved putative zinc fingers of

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the CCH class (Varnum et al, Mol. Cell. Biol. 11:1754 (1991); Taylor et al, Nucleic Acids Res. 19:3454 (1991); Gomperts et al, Oncogene 5:1081 (1990); Ma et al, Oncogene 9:3329 (1994)). In addition, the protein has been shown to bind Zn<sup>++</sup> and has been localized to the cell nucleus in mouse fibroblasts (DuBois et al, J. Biol. Chem. 265:19185 (1990)), suggesting that it may be a transcription factor. Serum or other mitogen stimulation of quiescent fibroblasts causes rapid serine phosphorylation and nuclear to cytosolic translocation of TTP (Taylor et al, J. Biol. Chem. 270:13341 (1995); Taylor et al, Mol. Endocrinol. 10:140 (1996)), both of which are likely to modulate its function in cells.

In the adult mouse, TTP mRNA is highly expressed in lung, intestine, lymph node, spleen, and thymus, with lower expression in adipose tissue, kidney, and liver, and negligible expression in skeletal muscle and brain (Lai et al, J. Biol. Chem. 265:16556 (1990); DuBois et al, J. Biol. Chem. 265:19185 (1990)). In the thymus, TTP mRNA is highly expressed in both cortical and medullary thymocytes, while in the spleen, it is highly expressed in B and T lymphocytes within the white pulp, and is expressed at somewhat lower levels in the myeloid cells of the red pulp and endothelial cells of the high endothelial venules. In addition, TTP is constitutively expressed in several types of blood cells, particularly neutrophils, macrophages and B and T lymphocytes. The function of TTP in normal vertebrate physiology, however, was heretofore unknown.

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OBJECTS AND SUMMARY OF THE INVENTION

It is a general object of the invention to provide a method of modulating cellular levels of TNF $\alpha$ .

5 It is a specific object of the invention to provide a method of treating diseases and disorders associated with TNF $\alpha$  excess.

It is a further object of the invention to provide a method of identifying an individual at  
10 increased risk to the effects of TNF $\alpha$  excess.

It is another object of the invention to provide a method of selecting compounds for there ability to inhibit TNF $\alpha$  production, processing or secretion.

It is a further object of the invention to  
15 provide a TTP-deficient non-human mammal.

These objects are met by the present invention.

In one embodiment, the present invention relates to a method of inhibiting TNF $\alpha$  production, processing or secretion in a mammal. The method comprises  
20 increasing the level of TTP, or a TNF $\alpha$  production, processing or secretion-inhibitory polypeptide fragment thereof, in the mammal so that the inhibition is effected.

In a further embodiment, the present invention  
25 relates to a method of treating an effect of excess TNF $\alpha$  in a mammal. The method comprises administering to the mammal TTP, or polypeptide fragment thereof that inhibits TNF $\alpha$  production, processing or secretion, or agent that enhances a TNF $\alpha$  production,  
30 processing or secretion-inhibitory effect of TTP, in an amount sufficient to effect the treatment.

In yet another embodiment, the present invention relates to a method of identifying a subject susceptible to a TNF $\alpha$  associated disease or disorder.  
35 The method comprises isolating a DNA-containing

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biological sample from the subject, locating the TTP gene present in the DNA and comparing the nucleic sequence of the TTP gene with a wild-type TTP encoding sequence and thereby determining whether the TTP gene includes a mutation that renders the subject susceptible to the disease or disorder.

In a further embodiment, the present invention relates to a method of identifying a subject susceptible to a TNF $\alpha$  associated disease or disorder.

The method comprises

i) isolating a biological sample from the subject,

ii) contacting the sample with a TTP binding partner under conditions such that complexation between TTP and the binding partner can occur, and

iii) detecting the presence or absence of the complexation, or comparing the extent of the complexation with a control sample comprising wild-type TTP.

In another embodiment, the present invention relates to a method of screening a compound for its ability to enhance the ability of TTP to inhibit TNF $\alpha$  production. The method comprises

i) contacting the compound with a sample comprising an expression construct comprising a TNF $\alpha$  encoding sequence, in the presence of TTP or TNF $\alpha$  production-inhibitory polypeptide fragment thereof, under conditions such that the TNF $\alpha$  encoding sequence can be expressed, and

ii) determining the level of expression of the TNF $\alpha$  encoding sequence and comparing that level to a level of expression obtained in the absence of the compound.

In yet a further embodiment, the present invention relates to a method of screening a compound

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for its ability to enhance a TNF $\alpha$  transcription-repressor effect of TTP. The method comprises

5           i) contacting the compound with a sample comprising an expression construct comprising a TNF $\alpha$  promoter sequence operably linked to an encoding sequence, in the presence of TTP or TNF $\alpha$  production-inhibitory polypeptide fragment thereof, under conditions such that the encoding sequence can be expressed, and

10          ii) comparing the level of expression of the encoding sequence obtained to a level of expression obtained in the absence of the compound.

          In another embodiment, the present invention relates to a method of screening a compound for its ability to enhance a TNF $\alpha$  mRNA translation-inhibitory effect of TTP. The method comprises

15           i) contacting the compound with a sample comprising TNF $\alpha$  mRNA, in the presence of TTP or a TNF $\alpha$  translation-inhibitory fragment thereof, under conditions such that translation of the TNF $\alpha$  mRNA can be effected, and

20           ii) determining the level of translation of the TNF $\alpha$  mRNA and comparing that level of translation of the TNF $\alpha$  mRNA to a level of translation of TNF $\alpha$  mRNA obtained in the absence of the compound.

25           In yet a further embodiment, the present invention relates to a TTP-deficient non-human mammal.

          In another embodiment, the present invention relates to a method of screening or testing a compound for its ability to treat a symptom of excess TNF $\alpha$ . The method comprises administering the compound to a TTP-deficient non-human mammal and monitoring the effect of the compound on the symptom.



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Further objects and advantages of the invention will be clear from the description that follows.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Expression of TTP mRNA (a) and protein (B). (a) Total RNA was isolated from the indicated tissues of (+/+), (+/-), and (-/-) mice and subjected to northern blotting with a TTP cDNA probe. Each lane contains 15µg of total cellular RNA. The lower band represents endogenous TTP mRNA, whereas the upper band represents the TTP-neo fusion RNA. (b) Primary embryonic fibroblasts were isolated from three different (+/-) (A-C) and three (-/-) (D-F) embryos. The cells were serum-deprived for 14 h, and then exposed to [<sup>35</sup>S]-cysteine for 2 h and 20% fetal calf serum for an additional 2 h. Lysates from these cells were used for immunoprecipitation with an antibody that recognizes the amino-terminus of TTP, and the immunoprecipitated proteins were separated on a 9% acrylamide SDS gel and an autoradiograph prepared. The positions of molecular weight standards are indicated. TTP migrates at about 43 kDa. Immunoprecipitated proteins from the same samples were also separated on a 20% acrylamide SDS gel; no truncated amino-terminal TTP fragment was detected in the (-/-) samples.

Figure 2. Growth curves of two -/- and three +/- or +/- littermates. Weekly weights of one litter of five pups were determined; genotypes of each mouse are indicated to the right.

Figure 3. Flow cytometric analysis of (a) Gr-1<sup>+</sup> neutrophils, (b) Ly-5<sup>+</sup> lymphocytes, (c) Thy-1<sup>+</sup> lymphocytes, and (d) F4/80<sup>+</sup> monocytes/macrophages, in

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peripheral blood adult (+/+) and (-/-) mice. Cell surface analysis was performed using a Becton Dickinson FACStar Plus flow cytometer and the accompanying software. Shown are the results from representative mice. The average percent positive cells and average absolute number of positive cells per mm<sup>3</sup> ( $\pm$  SEM) from 5-7 adult mice were: Gr-1: (+/+) 23 $\pm$ 4%, 1.13 $\pm$ 0.27X10<sup>3</sup>; (-/-) 48 $\pm$ 2%, 4.36 $\pm$ 0.72x10<sup>3</sup>; Ly-5: (+/+) 26 $\pm$ 3%, 1.36 $\pm$ 0.28X10<sup>3</sup>; (-/-) 6 $\pm$ 1%, 0.71 $\pm$ 0.15x10<sup>3</sup>; Thy-1: (+/+) 33 $\pm$ 6%, 1.67 $\pm$ 0.53x10<sup>3</sup>; (-/-) 33 $\pm$ 3%, 3.41 $\pm$ 0.39x10<sup>3</sup>; F4/80: (+/+) 5 $\pm$ 1%, 0.29 $\pm$ 0.05x10<sup>3</sup>; (-/-) 8 $\pm$ 2%, 0.87 $\pm$ 0.26x10<sup>3</sup>. Similar analyses from spleen and bone marrow are discussed in the text.

Figure 4. Effect of TNF $\alpha$  antibody injections on body weights. As described in the text, TTP -/- mice from seven litters (each litter is labeled A-G) were injected at weekly intervals starting on day 10 of age with either anti-TNF monoclonal antibodies (AB; open circles) or PBS (closed circles). Body weights were measured at weekly intervals and are shown here. Littermates of the TTP +/+ and +/- genotypes are indicated by the lines without symbols. TTP -/- mice from litters B, C and D that were injected with PBS died before completion of the experiments, which consisted of nine weekly injections. The means  $\pm$  SEM of the six antibody-injected animals and the six PBS-injected animals are shown in H; the normal range (mean  $\pm$  S.D.) from all of the control littermates (n = 25) is indicated by the dashed lines in H. The differences between the AB and PBS means were significant (p < 0.001 using Student's t test) at each time point after four weeks.

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DESCRIPTION OF THE INVENTION

The present invention results from the realization that TTP regulates effective levels of TNF $\alpha$  in animals, eg mammals. As indicated above, TNF $\alpha$  has been implicated in the pathology of a variety of neoplastic diseases, immune disorders and infections. Accordingly, the ability of TTP to regulate TNF $\alpha$  levels is of considerable pharmaceutical importance.

The identification of this regulatory activity of TTP makes possible methods of screening compounds for their ability to enhance the TNF $\alpha$  production-inhibitory activity of TTP, as well as their ability to enhance the effect of TTP on TNF $\alpha$  processing and secretion. It also makes possible methods of identifying susceptibility to TNF $\alpha$ -associated diseases, including inflammatory conditions and sepsis. Further, the demonstration of TTP as an inhibitor of TNF $\alpha$  production, processing or secretion makes possible new modes of therapy for diseases or disorders mediated by or exacerbated by TNF $\alpha$ .

It will be appreciated that the preferred subject of the invention is a human, however veterinary uses are also contemplated.

Compound Screens

The natural ability of TTP to inhibit cellular production, processing or secretion of TNF $\alpha$  permits the screening of test compounds for their ability to enhance this inhibitory effect. Using appropriate screens, compounds can be identified that potentiate the ability of TTP to inhibit TNF $\alpha$  production, processing or secretion, independent of the mechanism by which this inhibition occurs.

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In one example of a screening system of the invention, a host cell is cotransfected with a vector encoding TTP and a vector containing the TNF $\alpha$  gene, for example, operably linked to a reporter gene.

5 Examples of commonly used reporter genes include chloramphenicol acyltransferase (CAT), alkaline phosphatase, luciferase, growth hormone, thymidine kinase, etc. Potential host cells include fibroblasts, HeLa cells, and macrophage and  
10 lymphocyte cells lines. Transfection can be effected using art-recognized techniques.

Using this type of system, test compounds can be assayed for their ability to shift the TTP dose response curve to the left, that is, to decrease the  
15 dose at which TTP inhibits TNF $\alpha$  production (or processing or secretion) as measured, for example, by the reporter expression. Compounds identified as being capable of enhancing (directly or indirectly) the TTP inhibitory activity can then be further  
20 assayed, using standard protocols, for stability, toxicity etc.

While the foregoing screen can be used to assay test compounds for their ability to decrease the dose at which TTP inhibits TNF $\alpha$  production (or processing  
25 or secretion), various other screens can be devised based on the mechanism by which TTP exerts its inhibitory effect. For example, if TTP inhibits TNF $\alpha$  gene transcription, then a screen can be used in which sequences within the TNF $\alpha$  gene promoter are  
30 linked to a reporter gene. Such constructs can be used in cell transcription studies or cell-free transcription assays in the presence or absence of TTP and the test compound. Similarly, if TTP inhibits TNF $\alpha$  mRNA translation, test compounds can be  
35 added, for example, to cell-free translation assays in the presence or absence of TTP and TNF $\alpha$  mRNA. The

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rate of synthesis of the TNF $\alpha$  protein can then be determined. Other screens can also be used, dependent on the biochemical site of action of TTP (eg gene transcription, mRNA translation, protein processing or protein secretion).

Examples I-V below describe an animal model of TTP deficiency produced using gene targeting in murine embryonic stem cells. The resulting mice, while appearing normal at birth, develop a phenotype consistent with whole body TNF $\alpha$  excess. This animal model (as well as other such TTP-deficient animal (eg mammalian) models) can be used to screen and/or test agents for their ability to prevent/treat the effects of excess TNF $\alpha$ . The test agent can be administered to the model animal (eg orally or by injection) in accordance with standard test protocols and the effects on animal growth and phenotype monitored. Compounds that prevent the development of one or more aspects of the phenotype of TTP-deficient animals can then be further tested for pharmaceutical acceptability using standard protocols.

#### Detection/Diagnosis

The TTP deficiency produced in Examples I-V causes a severe syndrome of wasting and arthritis that is often lethal. Partial deficiencies resulting from the production of mutant forms of TTP, while perhaps non-lethal, can be expected to increase susceptibility to TNF $\alpha$  associated diseases and disorders, including infections, and autoimmune disorders (likewise, heterozygosity). The availability of the amino acid sequence of TTP (eg the human TTP sequence, Taylor et al, Nucl. Acids Res. 19:3454 (1991)) and its encoding sequence (eg GenBank accession number M63625) makes possible methods of identifying and diagnosing individuals at

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increased risk, for example, for inflammatory conditions or sepsis following infection.

General mutation screening of the TTP gene to identify subjects at increased risk can be performed by such methods as direct sequencing of cDNAs from candidate patients. Alternatively, approaches based on the cytogenetic mapping of the human gene to chromosome 19 q 13.1 (Taylor et al, Nucl. Acids Res. 19:3454 (1991)) can be used. For example, sequences from the human cDNA or genomic DNA can be used to locate the TTP gene (ZEP36) on a physical map of chromosome 19 (see, for example, Garcia et al, Genomics 27:52 (1995)). One or more polymorphic loci in the immediate vicinity of the gene can then be identified. Polymerase chain reaction (PCR) primers, for example, can be used to screen genomic DNA from populations (eg members of multiple families with rheumatoid arthritis) for polymorphisms closely linked to the TTP gene. Direct DNA sequencing of genomic DNA from likely candidate patients can then be accomplished using, for example, PCR sequencing strategies.

In an alternative approach, a biological sample can be obtained from a subject suspected of being at increased risk and the sample examined for the presence of a mutated form of TPP. Biological samples suitable for use in this regard include blood cells and transformed cell lines derived therefrom, lung lavage fluid, ascites fluid, etc. Tissue samples can also be used, including samples from liver, kidney intestine, spleen, lymph nodes, etc. Detection of a mutant form of the protein can be effected by isolating the protein from the sample and determining its amino acid sequence or by contacting the protein (in purified or semi-purified form) with a binding partner (eg an anti-wild type TTP antibody

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or TNF $\alpha$  promoter sequence) and determining whether complexation occurs. Complexation (or lack thereof) can be established using any of a variety of art recognized techniques (eg use of a labeled binding partner, use of a binding partner bound to a solid support, etc). Mutant forms of the protein can be expected to have altered (eg decreased) affinity for the binding partner. In addition, abnormalities in the size, charge or relative amount of the protein identified, for example, by means of a binding partner (such as an antibody) indicate a mutation in the TTP gene. Mutations can be confirmed by routine sequencing of the gene.

The identification of mutant forms TTP or its encoding sequence makes possible the identification of subjects likely to benefit from increased monitoring or therapeutic intervention.

#### Therapy

The present invention contemplates the use in gene therapy regimens of DNA sequences encoding TTP or portions thereof encoding TNF $\alpha$  production (processing or secretion)-inhibiting polypeptides. The encoding sequences can be present in a construct which, when introduced into target cells, results in expression of the TTP encoding sequences and thus production of the TNF $\alpha$  production (processing or secretion)-inhibitor.

For gene therapy to be practical, it is desirable to employ a DNA transfer method that: (1) directs the therapeutic sequence into specific target cell types (2) is highly efficient in mediating uptake of the therapeutic polynucleotide into the target cell population, and (3) is suited for use *ex vivo* or *in vivo* for therapeutic application.

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Delivery of the TTP gene (or portion thereof encoding a TNF $\alpha$  production (processing or secretion)-inhibitor polypeptide) can be effected using any of a variety of methodologies. Presently available  
5 methodologies include transfection with a viral vector (eg retroviral vector or adenoviral vector) and fusion with a lipid. Other techniques are also available, many employing selectable markers to improve transfection efficiency. The technique  
10 selected depends upon the particular situation.

Retroviral vectors can be used to effect high efficiency gene transfer into replicating cells and such vectors are particularly suitable for use where  
15 target cells are present in a body compartment, such as brain and liver or epithelial surfaces such as lung, bladder or colon. Adenovirus vectors are advantageous from the standpoint that they have the potential to carry larger insert polynucleotide sequences than retroviral vectors and they have the  
20 ability to infect non-replicating cells. Further, they are suitable for infecting tissues *in situ*, especially in the lung. Adenoassociated viruses, which integrate, can also be used, as can other viral systems depending on the target site, including  
25 hepatitis virus when liver is the target tissue. Consistent with this approach, TTP sequences can be transfected into autologous bone marrow progenitor or stem cells, and those cells can then be transplanted back into the original donor, for example, after  
30 selection for cells expressing the transfected TTP sequences. Similar approaches are contemplated in the treatment of rheumatoid arthritis (Chernajorsky et al, Brit. Med. Bull. 51:503 (1995); Kiem et al, Curr. Opin. Oncol. 7:107 (1995)). (See also Morgan



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and Anderson, Annu. Rev. Biochem. 62:191 (1993) and Mulligan, Science 260:926 (1993) for further details relating to use of viral vectors for gene therapy.)

Another gene transfer method suitable for use in the present invention is the physical transfer of plasmid DNA in liposomes directly into target cells. Liposome-mediated DNA transfer has been described by various investigators (Liu et al, Gene Therapy 1:7 (1994); Huxley, Gene Therapy 1:7 (1994); Miller and Vile, FASEB J. 9:190 (1995)).

Essentially any suitable DNA delivery method can be used in the context of the present invention. Ex vivo transfection using viral vectors, however, may be preferred in certain settings. Use of the TTP gene truncated at the 3' untranslated region may serve to make the mRNA more stable. Alternatively, a TTP cDNA operably linked to a cell specific promoter can be used. In any case, transfection of hematopoietic marrow progenitors or stem cells ex vivo and reintroduction by bone marrow transplantation can be effected.

The nucleic acid-containing compositions of the invention can be stored and administered in a sterile physiologically acceptable carrier. The nucleic acid can be present in combination with any agent which aids in the introduction of the DNA into cells.

Various sterile solutions may be used for administration of the composition, including water, PBS, etc. The concentration of the DNA will be sufficient to provide a therapeutic dose.

Actual delivery of the gene sequence, formulated as described above, can be carried out by a variety of techniques including direct injection, administration to the lung and other epithelial

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surfaces, intravenous injection and other physical methods.

The present invention also contemplates the use of TTP, and TNF $\alpha$  production (processing or secretion)-inhibitor polypeptide fragments thereof, as a pharmaceutical agent to effect suppression of TNF $\alpha$  biosynthesis, processing or secretion. Polypeptides can be made using commonly used and widely available techniques for the synthesis of synthetic peptides. The TTP protein or polypeptide fragments thereof can be synthesized recombinantly using common expression systems such as *E. coli*, baculovirus, Cos cells, etc. The protein/polypeptide can then be purified and used, for example, for injection or infusion as with many protein drugs currently available for clinical use. Alternatively, TTP can be isolated from natural sources, using art recognized techniques.

The TTP protein, or fragment thereof, can be administered by any appropriate means to achieve the effect sought (eg treatment of Type I diabetes, systemic lupus erythematosus, rheumatoid arthritis or other inflammatory condition, tumor, infection, or the like). Parenteral administration is preferred, for example, periodic subcutaneous, intramuscular, intravenous, intraperitoneal or intranasal routes can be used using either bolus injection or gradual infusion. Alternatively, topical or oral administration can be used.

The optimum dosage administered will vary with the subject, the protein/polypeptide and the effect sought. Appropriate doses can be readily determined by one skilled in the art.

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Compositions suitable for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions or emulsions. Compositions can be present in other dosage unit form (eg tablet or capsule). Compositions suitable for topical administration can be in the form of a cream, gel, ointment, lotion or foam.

The compositions comprise the TTP protein/polypeptide, in an amount effective to 10 achieve the desired result, together with a pharmaceutically acceptable carrier.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

## 15 EXAMPLES

The following experimental details pertain to the Examples I-V which follow.

Generation of TTP-deficient mice. A TTP insertion targeting vector was created by first isolating a 3.8 kb Zfp-36 (TTP genomic) clone from a SV129 library (Stratagene, La Jolla, CA) using a mouse TTP cDNA probe (Lai et al, J. Biol. Chem. 265:16556 (1990)); this fragment was cloned into the SalI site of BS+ (Stratagene). A 1.14 kb XhoI-BamHI neo fragment from pMC1PolA (Stratagene) was then ligated into the TTP SstI site (1 kb downstream of the initiator ATG) in pBS+/TTP. Next, a 4.9 kb SalI TTP-neo fragment from pBS+/TTP-neo was cloned into the SalI site of pSP73 (Promega, Madison, WI), into which two thymidine kinase genes (ClaI-BamHI and HindIII-XhoI fragments of pIC19R/MC1-TK) (Mansour et al, Nature 336:348 (1988)) had been cloned previously

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at the pSP73 ClaI-BamHI and HindIII-XhoI sites. This targeting vector was linearized with HindIII and electroporated into ES cells, which were then used to generate chimeric mice according to established methods (Koller et al, Proc. Natl. Acad. Sci. USA 86:8932 (1989)).

Northern blot analysis. Dissected tissues were rapidly frozen in liquid nitrogen, pulverized in liquid nitrogen, and then homogenized in a guanidinium thiocyanate solution, as described previously (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)). Total cellular RNA was isolated from the tissue lysate using an established acidic phenol extraction procedure (Chomzynski and Sacchi, Anal. Bioch. 162:156 (1987)). 15 µg RNA samples were separated by electrophoresis in 1.2% agarose/formaldehyde gels and used for northern blotting (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)) with a [<sup>32</sup>P]-labeled mouse cDNA probe (Lai et al, J. Biol. Chem. 265:16556 (1990)).

Cell culture and immunoprecipitation. Primary embryonic fibroblasts were prepared (Robertson, Robertson, E.J., ed. (IRL Press, Oxford) pp. 77-78 (1987)) from 14-17 day mouse embryos that had been generated from TTP(+/-) mouse matings. To identify (+/-) and (-/-) cell lines, DNA was isolated from the cells (Koller et al, Proc. Natl. Acad. Sci. USA 86:8932 (1989)), digested with EcoRI and subjected to Southern blot analysis (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)) using as a probe a 2.4 kb BstEII-HindIII TTP gene fragment (Taylor et al, Nucl. Acids Res. 19:3454 (1991)). Using this strategy, the 7.5 kb EcoRI fragment that resulted from a targeted TTP allele was easily distinguishable from the 10 kb EcoRI fragment that resulted from a wild-type TTP allele.

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Cell labeling and immunoprecipitation protocols have been described (Taylor et al, J. Biol. Chem. 270:13341 (1995)). Briefly, confluent 60 mm plates of cells were serum-deprived for 24 h in DMEM supplemented with 1% (w/v) bovine serum albumin, and then exposed to [<sup>35</sup>S]-cysteine for 2 h and 20% (v/v) fetal calf serum for an additional 2 h. Next, the cells were lysed by brief sonication in a buffer containing 1% (w/v) nonidet P-40, 5mM EDTA, 0.15M NaCl, and 50mM Tris, pH8.3; protein was precipitated with an immunopurified polyclonal antiserum that recognized the 24 amino-terminal amino acids of TTP (Taylor et al, J. Biol. Chem. 270:13341 (1995)). Precipitated proteins were separated on 9% or 20% polyacrylamide SDS gels, which were dried and used for autoradiography.

Myeloid progenitor cell assays. Assays were performed on femoral bone marrow, peripheral blood and spleen from +/+ and -/- mice at 33 days of age (young mice) or 6.5 to 12 months of age (adult mice). These were performed as described (Cooper et al, Exp. Hematol. 22:186 (1994)). Marrow, spleen and blood cells were respectively plated at concentrations of  $2.5 \times 10^4$ ,  $2.5 \times 10^5$  and  $1.0 \times 10^5$  cells/ml in 1.0% methylcellulose culture medium with 30% fetal bovine serum (Hyclone, Logan, UT), 0.1 mM hemin, 1 U/ml recombinant (r) human (hu) erythropoietin (Epo, Amgen Corp., Thousand Oaks, CA), 5% vol/vol pokeweed mitogen mouse spleen cell conditioned medium (PWMSCM), and 50 ng/ml r murine (mu) steel factor (SLF; Immunex Corporation, Seattle, WA). Colonies were scored after 7 days incubation at 5% CO<sub>2</sub> and lowered (5%) O<sub>2</sub>. Calculation of the absolute numbers of progenitors per organ was based on the nucleated cellularity and colony counts for CFU-GM, BFU-E and CFU-GEMM in each organ for each individually assessed

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mouse. Cultures were also set up in methylcellulose or 0.3% agar (10% fetal bovine serum) in the presence or absence of different concentrations of Epo, rmu granulocyte-macrophage colony stimulating factor, rhu granulocyte colony stimulating factor, rhu macrophage colony stimulating factor (Immunex Corp) or PWMSCM with or without rmu SLF or rhu Flt-3 ligand (Immunex Corp) to assess the sensitivity of cells to stimulation by single or multiple cytokines.

Histological analysis. Mouse tissues were immersed in Bouin's fixative for 2 to 4 days, and then washed for several days in 70% (v/v) ethanol at room temperature. When required, tissues were decalcified following Bouin's fixation by immersing in 12.5% (w/v) sodium citrate and 25% (v/v) formic acid for 24 h, rinsing in running water for 24 h, and then washing for several days in 70% ethanol, all at room temperature. Fixed tissues were then embedded for paraffin sectioning; 5-7  $\mu$ m sections were stained with hematoxylin and eosin by standard methods, then photographed with a Nikon Opiphot-2 photomicroscope and Kodak Ektar 100 film.

Renal pathology was evaluated at 5 months of age. One kidney was fixed, embedded in paraffin and sectioned as described above prior to staining with hematoxylin and eosin, Congo red and periodic acid Schiff (PAS) (Tse, Mishell and Shiigi, eds. (W.H. Freeman and Company, New York), pp. 201-205 (1980)). The other kidney was quick frozen in OCT embedding compound on dry ice, and frozen sections were prepared for immunofluorescent microscopy using fluorescein-conjugated goat anti-mouse IgG or IgM as described (Andrews et al, Vet. Pathol. 31:293 (1994)).

Glomerular disease was graded by a pathologist blinded as to the genotype of origin of the kidney

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sections. Scores were determined using a grading system that assigns 0-3+ scores for proliferation, necrosis, crescent formation, vasculitis, and inflammatory infiltrate. IgG and IgM deposition were graded 0-3+ on the fluorescent slides by the same pathologist.

Fluorescent activated cell sorter (FACS) analysis. Peripheral blood cells were obtained by capillary tube bleeding from the eye orbit; bone marrow cells were obtained by flushing dissected femurs with 2mL ice cold RPMI 1640 medium followed by gentle pipeting to disperse the cells; and splenocytes and thymocytes were obtained by macerating dissected tissues with the plunger of a disposable 1 ml syringe in ice-cold RPMI 1640 medium, and then isolating the cells by density centrifugation (Tse, Mishell and Shiigi, eds. (W.H. Freeman and Company, New York), pp. 201-205 (1980)). Some peripheral blood and bone marrow cells were stained with ACCUSTAIN Wright Stain (3WS10) as described by the manufacturer.

Analysis of cell surface phenotype was performed on the cell preparations according to previously described direct and indirect-immunofluorescence assays (Haynes et al, New Engl. J. Med. 304:1319-1323 (1981)), using a FACStar Plus Flow Cytometer and associated software (Becton Dickinson). The following directly conjugated monoclonal antibodies were used at saturating titers: Thyl.2 (anti-Thy-1, Becton Dickinson, Mountain View, CA), Ly-5 (anti-B220, Caltag, South San Francisco, CA) Lyt2 (anti-CD8, Becton Dickinson), L3T4 (anti-CD4, Becton Dickinson), OX-12 (anti-rat, Sera-Labs, Crawley Down, Sussex, England), and streptavidin-phycoerythrin (Pharmingen, San Diego, CA). PK-136 (American Type Culture Collection (ATCC) HB191) was purified from

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serum-free media (Gibco, Grand Island, NY) hybridoma culture supernatant using affinity chromatography over a Staphylococcal protein (A/G) column (Pierce, Rockford, NY), then fluorescein conjugated and used at saturating titer. The following hybridomas were cultured in serum-free medium and supernatant was used in indirect immunofluorescence flow cytometry assays using fluorescein-conjugated OX-12 as a secondary reagent: F4/80 (ATCC HB198), 14.8 (ATCC TIB164), Gr-1 (RB6-8CA, a gift of R.L. Coffman, DNAX, Palo Alto, CA), Ter119 (a gift of I.L. Weissman, Stanford University), and Y3-Ag1.2.3 (ATCC CRL 1631).

Evaluation of autoimmunity. DNA from calf thymus was purchased from the Sigma Chemical Co. DNA was dissolved in SSC (0.15M Na citrate, pH8) prior to purification by phenol extraction. Double-stranded DNA (dsDNA) was obtained by treating the DNA with S<sub>1</sub> nuclease while single-stranded DNA (ssDNA) was obtained by boiling for 10 min prior to rapid immersion in ice.

Sera obtained as described above were tested for reactivity to DNA antigens by ELISA as previously described (Gilkeson et al, J. Immunol. 151:1343 (1993)). Briefly, 96 well polystyrene plates were coated with DNA diluted to 5µg/ml in SSC. Antigens used in these assays were calf thymus dsDNA (dsDNA) and calf thymus single stranded DNA (ssDNA). After addition of DNA, plates were incubated for 2h at 37°C for ssDNA assays and 16 h at 37°C for dsDNA assays. Two-fold serial dilutions of sera in PBS-T (phosphate buffered saline containing 0.05% Tween 20) were then added to the plates starting at a 1/100 dilution. Following incubation, peroxidase conjugated goat-anti-mouse IgG was added. 3,3', 5,5' Tetramethylbenzidine (TMB) in 0.1M citrate (pH4) with



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0.015%  $H_2O_2$  was added for color development.  $OD_{380}$  absorbance was determined by a microtiter plate reader (Molecular Dynamics, Menlo Park, CA).

Rheumatoid factor (RF) activity in the sera was also determined by ELISA. For IgM RF assays, microtiter plates were coated with mouse IgG (Sigma) at 1  $\mu g/ml$  in PBS; for IgG RF assays, plates were coated with 1  $\mu g/ml$  of rabbit IgG in PBS. After blocking with 1% BSA in PBS, sera were added in dilutions beginning at a 1/100 dilution. Peroxidase conjugated goat anti-mouse IgG ( $\gamma$  chain specific) or goat anti-mouse IgM ( $\mu$  chain specific) were added to the plates followed by the substrate. After color development, absorbance at  $OD_{380}$  was determined.

*Crithidia luciliae* assays for anti-dsDNA were performed as suggested by the manufacturer (Kallestad, Austin, TX). Sera were tested at 1/20 and 1/50 dilutions.

Antinuclear antibody assays were performed as suggested by the manufacturer (Zeuss Scientific, Raritan, NJ). Sera were tested at 1/20 and 1/50 dilutions.

*TNF $\alpha$  antibody administration.* To test the possible role of  $TNF\alpha$  in the development of the TTP-deficient phenotype, six -/- mice received weekly intraperitoneal injections of a hamster monoclonal antibody (TN3-19.12) that is specific for mouse  $TNF\alpha$  (Sheehan et al, J. Immunol. 142:3884 (1989)); a general gift from Dr. Robert D. Schreiber, Washington University School of Medicine) and six -/- mice received an equivalent volume of PBS. The first injection occurred when the animals were 10 days of age, and continued at weekly intervals for a total of nine injections. The first two injections were 125  $\mu g$  of antibody in 50  $\mu l$  PBS, and the last seven injections were 250  $\mu g$  of antibody in 100  $\mu l$  of PBS.

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One week after the final injection, the animals were killed with CO<sub>2</sub>, and blood and tissues were harvested for blood counts and histology as described above.

## EXAMPLE I

## 5                      Generation of TTP (-/-) Mice

A targeting vector was constructed that contained 3.8kb of the gene encoding TTP, Zfp-36 (Taylor et al, Nucl. Acids Res. 19:3454 (1991)), in which a neomycin resistance gene (neo) was inserted  
10 into the protein-coding portion of the second exon. Insertion of this sequence introduced multiple stop codons upstream of the sequences encoding the two putative zinc fingers, precluding synthesis of functional TTP protein. Using this targeting vector  
15 and established experimental methods (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932 (1989)), TTP-deficient mice were generated. Among the first 492 offspring of heterozygous (+/-) crosses, 126 (26%) were homozygous wild-type (+/+), 267 (54%) were  
20 heterozygous (+/-), and 99 (20%) were homozygous null (-/-), indicating that there was no substantial embryonic lethality associated with the (-/-) genotype.

Northern analysis of tissues from (+/-) mice  
25 revealed that the endogenous TTP mRNA signal was decreased by about 50%, and that a TTP/neo fusion mRNA had been generated (Fig. 1a). No endogenous TTP mRNA was detected in tissues from a (-/-) mouse, but the TTP/neo fusion mRNA signal was increased over  
30 that seen in the +/- mice. Because the neo portion of the TTP/neo fusion mRNA contains many termination codons, a complete TTP/neo translation product should not be made; however, translation of an amino-

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terminal TTP fragment could have occurred. To test this possibility, primary embryonic fibroblasts were generated from both (+/-) and (-/-) embryos, and immunoprecipitations were performed on lysates from these cells, using an antibody (Taylor et al, J. Biol. Chem. 270:13341 (1995)) directed at the amino-terminus of TTP (Fig. 1b). Although TTP was readily detectable in (+/-) cells, neither intact TTP nor a truncated amino-terminal TTP fragment could be detected in the (-/-) cells (Fig. 1b).

The expression of two other mRNAs that encode related CCH zinc finger proteins was also measured to determine if their expression was compensatorily increases in TTP-deficient mice; however, no change in expression of either TIS11B (CMG1) (Varnum et al, Oncogene 4:119 (1989), Taylor et al, Nucl. Acids Res. 19:3454 (1991)) or TIS11D (Varnum et al, Mol. Cell Biol. 11:1754 (1991)) was noted.

#### EXAMPLE II

##### Histological Characteristics of TTP (-/-) Mice

The (-/-) mice appeared normal at birth, but their rate of weight gain began to decrease compared to littermates between one and eight weeks after birth (Fig. 2). This failure of weight gain and eventual cachexia was one of the most striking characteristics of the phenotype, and occurred in essentially all of the mice to varying degrees (Fig. 2). They also developed patchy alopecia, dermatitis, arthritis, and conjunctivitis. Although all (-/-) mice eventually developed the syndrome, the degree to which they were affected was variable. 34% (of 56) were severely affected and died before reaching seven months of age. The remaining (-/-)

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mice were less severely affected, but nevertheless, 68% (of 37) displayed dermatitis, 88% arthritis, and 72% conjunctivitis by seven months of age. Survival of a (-/-) mouse was 16 months or more.

5       Histological examination of the (-/-) mice demonstrated several characteristic abnormalities. In the calvarial skin of seven-month old littermate mice, there was orthokeratotic hyperkeratosis in many areas, with an infiltration of neutrophils in the  
10       epidermis, and marked acanthosis. There was also diffuse inflammation in the underlying dermis, characterized by accumulation of many neutrophils and fewer lymphocytes, plasma cells, and macrophages. The inflammatory infiltrates extended to the deep  
15       dermal margins of the tissue, and in some cases, to the underlying skeletal muscle. Large numbers of Gr-1<sup>+</sup> neutrophils were present in both the epidermis and the dermis, while small foci of CD3<sup>+</sup>, TCR $\alpha\beta$ <sup>+</sup>, and Thy1.2<sup>+</sup> lymphocytes, 75% of which were of the CD4<sup>+</sup>  
20       subset, were also present in the dermis. A striking finding was that subcutaneous fat was essentially absent as was mesenteric and epididymal fat.

      The epidermis of the eyelid was also thickened, was the palpebral conjunctiva. Neutrophils  
25       infiltrated the dermis beneath both the eyelid and the conjunctiva. In addition, there were relatively few mucous cells along the surface of the conjunctiva in the -/- mice.

      In most joints in both the front and rear paws  
30       of the (-/-) mice at seven months of age, the synovium was markedly inflamed and thickened, with proliferating synovial cells extending well into the joint spaces; the synovium contained many Mac-1<sup>+</sup> macrophages and fewer Gr-1<sup>+</sup> neutrophils, CD3<sup>+</sup>, TCR $\alpha\beta$ <sup>+</sup>,  
35       and Thy1.2<sup>+</sup> lymphocytes and plasma cells. In addition, there was apparent proliferation of

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synovial cells and pannus formation, which in some cases completely separated apposing joint surfaces. Erosion of articular cartilage by pannus and extensive bone destruction were common. The marrow cavities were densely filled with cells of the myeloid lineage, especially mature neutrophils, and there was marked osteolysis of the inner aspect of the cortical bones.

Several abnormalities were also noted in the hematopoietic systems of the (-/-) mice. Thymuses in adult (-/-) mice were hypoplastic and showed no cortical/medullary organization; the thymuses of four days post-pactum (-/-) mice were decreased in size by an average of 50%. Spleens of the (-/-) mice were enlarged by an average of 41%, and there was extensive splenic myeloid hyperplasia, with many metamyelocyte, bands, and segmented neutrophils present. The perirenal, submaxillary, and mesenteric lymph nodes were also often enlarged, again showing extensive extramedullary hematopoiesis, primarily granulopoiesis. There was a marked increase in the number of myeloid cells in the bone marrow, which appeared nearly white in contrast to the red marrow of the control animals. Essentially all of the marrow myeloid cells were strongly Gr-1<sup>+</sup>. Although the cellular architecture of the liver appeared normal in (-/-) mice, foci of necrotizing hepatitis were present that contained a mixed inflammatory exudate of neutrophils, macrophages, and lymphocytes. There was also an inflammatory abscess in the interventricular septum of one mouse.

Because antinuclear antibodies were present (see below), the kidneys were examined histologically by staining with hematoxylin and eosin, periodic acid Schiff (PAS), and Congo red, and immunologically, for the presence and absence of IgG and IgM. The tubular

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and interstitial architecture was essentially normal in the kidneys from the -/- mice, but the glomeruli manifested increased cellularity and increased PAS-positive mesangial matrix. There was also focal, segmental thickening of peripheral capillary loops, which were congested with erythrocytes. IgG and IgM staining of glomeruli were similar in the kidneys from the +/+ and -/- mice. Proteinuria was not increased in the -/- mice compared to control, nor were plasma BUN and creatine significantly elevated compared to control.

## EXAMPLE III

## Hematopoietic Cell Populations in TTP (-/-) Mice

To further characterize the hematopoietic abnormalities seen in the (-/-) mice, complete blood counts and flow cytometric analyses of leukocyte subsets were performed (Fig. 3). In the (-/-) mice, the total peripheral white blood cell count was elevated by more than two fold [ $10.5 \pm 1.3$  (SEM)  $\times 10^3$  (n = 7) per  $\text{mm}^3$  vs.  $5.0 \pm 0.8 \times 10^3$  (n = 6) per  $\text{mm}^3$ ]. There was a marked increase in myeloid cells, with sharp increases in the number of Gr-1<sup>+</sup> neutrophils and F4/80<sup>+</sup> macrophages in peripheral blood and spleen, and in the number of Gr-1<sup>+</sup> neutrophils in bone marrow. The marrow myeloid cells were karyotypically normal, suggesting that they had not undergone malignant transformation. There were also increases in the number of PK136 natural killer cells in both peripheral blood and spleen. Conversely, there were smaller, less consistent decreases in B and T (B220) lymphocyte percentages and absolute numbers in hematopoietic tissues. Ly-5<sup>+</sup> B lymphocytes were decreased in peripheral blood, but were normal in

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spleen. The number of Thy-1<sup>+</sup> T lymphocytes in peripheral blood and spleen was normal, but the number of Thy-1<sup>+</sup> cells in bone marrow was decreased by two-fold. The peripheral red blood cell count, hemoglobin, hematocrit, and platelet count were within normal ranges; however, the Ter119<sup>+</sup> erythroid cells in bone marrow were decreased in percentage by about two-fold, presumably secondary to the massive increase of myeloid cells in the bone marrow.

- Assays of hematopoietic progenitor cells per organ were performed on cells from spleen, bone marrow and peripheral blood of young (age 33 days) and older (6.5 to 12 months) mice. In the young mice, absolute numbers of granulocyte-macrophage progenitors (CFU-GM) from -/- mouse bone marrow were increased approximately two-fold compared to control (+/+ (n=3):  $30.8 \pm 3.6 \times 10^3$ /femur (mean  $\pm$  SEM); -/- (n=3):  $64.3 \pm 0.5 \times 10^3$ /femur,  $p < 0.0025$  using Student's t test) whereas CFU-GM from spleen and peripheral blood were unchanged. Erythroid (BFU-E) and multipotential (CFU-GEMM) progenitors per spleen, femur and ml of peripheral blood were not significantly different ( $p > 0.05$ ) in the young -/- and +/+ mice. In the older mice, there were marked increases in myeloid progenitors (CFU-GM, BFU-E and CFU-GEMM) in spleen and peripheral blood but not bone marrow from the -/- compared to +/+ mice. Comparative progenitor cell values  $\times 10^3$  per spleen (-/- vs. +/+) were  $145 \pm 50$  vs.  $6 \pm 5$  for CFU-GM,  $115 \pm 51$  vs.  $7 \pm 4$  for BFU-E and  $10 \pm 4$  vs.  $0.2 \pm 2$  for CFU-GEMM. Comparative values per ml of blood were  $1530 \pm 781$  vs.  $46 \pm 39$  for CFU-GM,  $417 \pm 273$  vs.  $30 \pm 27$  for BFU-E and  $62 \pm 32$  vs.  $4 \pm 4$  for CFU-GEMM. Colonies from young or old -/- or +/+ mice did not form in vitro without addition of growth factors and no obvious differences in sensitivity of progenitor

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cells to stimulation of proliferation by single or multiple cytokines was apparent with bone marrow cells from -/- vs. +/+ mice.

5       Routine serum chemistries, including glucose, were within normal limits in the (-/-) mice, except for slightly decreased albumin and increases total serum globulin and  $\beta$ -globulin levels.

#### EXAMPLE IV

##### Autoantibodies

10       Rheumatoid factors (both IgG and IgM) and anti-Sm antibody titers were repeatedly normal in sera from the -/- mice. However, 3/4 of the sera from the -/- mice expressed high titers of antinuclear antibodies, with a homogenous pattern;  
15       these antibodies were not detected in 4/4 sera from the +/+ animals. The -/- sera (but not the +/+ sera) also contained antibodies to double-stranded DNA (mean +/- SEM of ELISA units from four -/- mice was 0.41 +/- 0.18 compared to 0.06 +/- 0.02 from four +/+  
20       mice;  $p = 0.11$  by Student's  $t$  test). This finding was confirmed by the Critihidia assay (4/4 -/- mice were positive, compared to 0/4 +/+ mice). Finally, sera from the -/- mice (but not from the +/+ mice) contained high titers of antibodies to single  
25       stranded DNA (mean +/- SEM ELISA units from four -/- mice was 1.49 +/- 0.30 compared to 0.22 +/- 0.05 from four +/+ mice;  $p = 0.006$  by Student's  $t$  test).



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## EXAMPLE V

TNF $\alpha$  Antibody Treatment

Because the phenotype of the TTP-deficient mice resembled that produced by chronic administration of TNF $\alpha$  (Keffer et al, EMBO J. 10:4025 (1991); Ulich et al, Res. Immunol. 144:347 (1993)), an attempt was made to prevent the development of the phenotype by treating the mice beginning at 10 days of age with nine weekly intraperitoneal injections of a hamster monoclonal antibody (TN3-19.12) that is specific for mouse TNF $\alpha$  (Sheehan et al, J. Immunol. 142:3884 (1989)). This antibody was originally thought to cross-react with TNF $\beta$  (Sheehan et al, J. Immunol. 142:3884 (1989)); however, subsequent work has shown that it does not neutralize the biological activity of this cytokine (R.D. Schreiber, personal communication). Four of six TTP -/- mice injected with PBS exhibited striking growth retardation, and one exhibited mild growth retardation (Fig. 4A). The sixth PBS-injected mouse died before growth retardation could become obvious (Fig. 4B); two others died before completion of the trial (Fig. 4). Three PBS-injected mice survived the trial, but were strikingly smaller than their wild-type littermates (Fig. 4). In contrast, five of six mice injected with the TNF $\alpha$  antibody maintained essentially identical growth curves to those of their +/- and +/- littermates (Fig. 4), whereas one exhibited slight growth retardation (Fig. 8B). The mean body weights of the six TTP -/- mice receiving the TNF $\alpha$  antibody were in the middle of the normal range throughout the trial, whereas the mean weights of those receiving PBS were significantly ( $p < 0.001$ ) lower than those

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receiving antibody at all time points after week 4 (Fig. 4H).

The antibody-injected mice also developed none of the cutaneous or joint stigmata associated with the -/- syndrome. In addition, the -/- mice injected with TNF $\alpha$  antibodies did not display medullary myeloid hyperplasia, with marrow from these animals containing  $33.7 \pm 3.1\%$  (SEM) mature granulocytes and bands, compared to  $30.4 \pm 3.0\%$  for the wild-type animals ( $p = .49$ ). Every examined aspect of the TTP-deficient phenotype has been essentially normalized by the injection of TNF $\alpha$  antibody.

#### EXAMPLE VI

Bone Marrow Transplantation Reproduces  
TTP-Deficiency Syndrome in RAG-2 (-/-) Mice

##### Experimental details:

Mice: TTP (-/-) mice were generated as previously described (Taylor et al, Immunity 4:445 (1996)). Genotyping of offspring was performed by Southern blot analysis of tail DNA as described (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)), using as a probe an Sst-II/Sst-I 1.1 kb fragment of the genomic DNA; this contained 79 bp of the promoter, the entire first exon, the entire intron and 275 bp of the second exon (Lai et al, J. Biol. Chem. 270:25266 (1995)). RAG-2 (-/-) mice were obtained from a colony maintained at the Division of Laboratory Animal Resources, Duke University Medical Center, Durham, NC. All mice were maintained in autoclaved microisolator cages in a barrier facility, and fed with autoclaved food and acidified water.

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*Bone marrow transplantation:* Bone marrow was obtained from two male TTP (-/-) mice and their two male TTP (+/+) littermates at age six months. Bone marrow transplantation was performed as described (Linton et al, Science 267:1034 (1995)). Briefly, the mice were euthanized by CO<sub>2</sub> inhalation, and both femurs were aseptically removed. Bone marrow was flushed from the femurs with RPMI medium (GIBCO-BRL, Grand Island, NY) supplemented with 2% (v/v) FCS, 100 U/ml of penicillin, 100 µg/ml of streptomycin (P/S) (GEBICO-BRL) and 5 U/ml of heparin (Elkins-Sinn Inc., Cherry Hill, NJ). 10<sup>7</sup> total bone marrow cells in a final volume of 300 µl were injected intravenously through the tail vein into 10 RAG-2 (-/-) female mice (7-9 weeks old), 5 receiving marrow from TTP (-/-) mice [(-/-) recipient group] and 5 receiving marrow from TTP (+/+) mice [(+/+) recipient group]. Bone marrow cells from different donors were not pooled. Thus, two groups were established, one of 6 mice receiving the marrow from one pair of (-/-) and (+/+) mice, and another of 4 mice receiving the marrow from the other pair of mice. Mouse body weight was assessed weekly after the transplantation, and blood smears (tail bleeding) were performed monthly. One mouse each from the (-/-) and (+/+) recipient groups was analyzed 10 weeks after the transplantation. The remaining animals were analyzed at later time points, usually when the recipients of the (-/-) marrow were near death. Animals were euthanized by CO<sub>2</sub> inhalation, and careful autopsies were performed. When possible, blood was collected into heparinized tubes for cell counts. Non-anticoagulated venous blood (vena cava) was collected for blood smears and for serum. Blood smears were stained using the modified Wright stain Diff Quick Stain Set (Baxter Healthcare Corporation, McGaw Park, ILL). Tissues

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were fixed for 48 hr in Bouin's fixative and extensively washed in 70% (v/v) ethanol. Fixed tissues were then embedded in paraffin and 5  $\mu$ m sections were cut and stained with hematoxylin-eosin by standard techniques. Tissues containing bones were fixed in Bouin's for 48 hr, and then decalcified by immersion in 12.5% (w/v) sodium citrate/25% (v/v) formic acid for 24 hr, then washed in running water for 24 hr. They were then extensively washed in 70% ethanol and embedded in paraffin as for the soft tissues. Bone marrow was flushed from the femurs with RPMI/10% (v/v) FCS. Cells were deposited onto glass slides (cytopreps) using a cytocentrifuge (Shandon Inc., Pittsburgh, PA) and stained with the Diff Quick Stain Set. Imprints from spleen, lymph nodes and liver were prepared by sectioning the organ and briefly pressing the section onto a glass slide. Cells in these preparations were also stained with the Diff Quick Stain Set. Stained sections and cells were photographed using a Leitz Laborlux- 12 microscope (Ernst Leitz, Wetzlar GMBH, Germany) equipped with an Olympus PM-C35B camera (Olympus America Inc., Lake Success, NY).

*In situ hybridization histochemistry for presence of the Y chromosome:* Because marrow donors were male and recipients were female, it was possible to evaluate the presence of donor cells in the recipients by DNA in situ hybridization histochemistry using a Y chromosome probe. Tissue samples for this use were fixed for 24 hr in ice-cold ethanol/acetic acid (3:1) (v/v), and then extensively washed in cold 70% ethanol (v/v) before being embedded in paraffin. Bone marrow cytopreps were also fixed in ice-cold ethanol/acetic acid, and kept at -20°C until used. In situ hybridization

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histochemistry was performed using a mouse Y chromosome probe (Bishop et al, Nucl. Acids Res. 15:2959 (1987)) (provided by Dr. Colin E. Bishop (Baylor College of Medicine, Houston, TX)). The probe was labeled with digoxigenin with the DNA Labeling and Non-Radioactive Detection Kit (Boehringer Mannheim, Indianapolis, IN), following the instructions of the manufacturer. DNA in situ hybridization histochemistry was usually performed on bone marrow cells, but in selected cases, it was also performed on spleen, lymph node and liver sections. In situ hybridization was performed according to the protocol described by Keighren and West (Keighren et al, Histochem. J. 25:30 (1993)). The only modification was that bone marrow cytopreps were not treated with xylenes before rehydration in ethanol.

*Detection of autoantibodies:* Detection of anti-DNA and anti-nuclear antibodies was performed exactly as described previously (Taylor et al, Immunity 4:445 (1996)).

*Culture of peritoneal macrophages:* In order to investigate possible cellular sources of excess TNF $\alpha$  in the TTP (-/-) mice, studies were performed in mice prepared as described (Taylor et al, Immunity 4:445 (1996)) that were not involved in the marrow transplantation studies. Peritoneal macrophages were prepared from two TTP (-/-) and their two TTP (+/+) litter-mate mice at 3 months of age. The mice were euthanized by CO<sub>2</sub> inhalation, and 5-10 ml of serum-free DMEM (GIBCO-BRL) were injected in the peritoneal cavity. The abdomen was gently massaged and a longitudinal incision was performed in the abdominal wall to allow recovery of the injected medium. The peritoneal cavity was then washed with an additional

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5-10 ml of medium, to obtain the greatest yield of macrophages. Recovered cells were counted on a hemocytometer. Purity of the macrophage population was assessed by Giemsa stain and nonspecific esterase activity (Tucker et al, J. Immunol. Methods 14:267 (1977)). Macrophages were plated at  $2.5 \times 10^5$  cells/well in 96-well plates, in DMEM supplemented with 10% (v/v) FCS and different concentrations of LPS (Sigma Chemical Co., St Louis, MO) (0, 0.1, 1, 10, 100 ng/ml), and cultured for 24 hr. The supernatants were then assayed for  $\text{TNF}\alpha$  accumulation as described below.

Culture of fetal liver macrophages: Fetal liver macrophages were obtained from hematopoietic progenitors present in fetal liver. Pregnant TTP (+/-) females that had been mated with TTP (+/-) males were euthanized by  $\text{CO}_2$  inhalation on day 14-16 of gestation and the fetuses were removed. Fetal livers were carefully and aseptically removed and single cell suspensions were obtained by dispersing the tissue between two glass slides in harvesting medium (GIBCO-BRL) supplemented with 10% (v/v) FCS, 15 mM HEPES (pH 7.4), 0.2% (w/v) sodium bicarbonate, P/S and 2 mM glutamine. Fetal liver macrophages were prepared from these suspensions according to the method described by Warren and Vogel (Warren et al, J. Immunol. 134:982 (1985)) for bone marrow-derived macrophages, and cultured for two weeks in the presence of harvesting medium supplemented with 30% TKL cell (ATCC, Rockville, MD)-conditioned medium, prepared as described (Hume et al, J. Cell. Physiol. 117:189 (1983)). After two weeks in culture, cells were harvested with the neutral protease Dispase H (Boehringer Mannheim, Indianapolis, IN) [1.5 mg/ml in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  free Earle's balanced salt solution], as

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described (Warren et al, J. Immunol. 134:982 (1985)), except that the cells were resuspended in RPMI supplemented with 2 mM glutamine, P/S, 30 mM HEPES (pH 7.4) and 0.4% (w/v) sodium bicarbonate, using 10% (v/v) instead of 2% FCS. Purity of the preparations was assessed by morphology (Diff Quick Stain Set) and non-specific esterase activity. By these criteria, the cells obtained by this method were more than 90% macrophages. Macrophages were then plated at  $1.25 \times 10^6$  cells/ml on 96-well plates and incubated in 200  $\mu$ l of the same medium at 37°C for 24 hr before being stimulated for another 24 hr with increasing concentrations of LPS (0, 1, 10, 100, 1000 ng/ml). In another series of studies, cells were plated in 60 mm dishes, and incubated in the presence or absence of  $\mu$ g/ml LPS for 4 hr. In all cases, supernatants were harvested and assayed for TNF $\alpha$  accumulation by western blot, as described below. Cells from the 4 hr LPS stimulation were harvested and used for the measurement of TNF $\alpha$  and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, as described below.

*Culture of bone marrow-derived macrophages:*

Bone marrow-derived macrophages were prepared as described by Warren and Vogel (Warren et al, J. Immunol. 134:982 (1985)). Bone marrow cells from both humeruses and both femurs were isolated from five TTP (-/-) and five TTP (+/+) mice (age 6-7 months). Cells were cultured as described above for fetal liver macrophages, except that at day 1, cells were split in two T75 CM<sup>2</sup> flasks (Becton Dickinson, Franklin Lakes, NJ), instead of one, as usually done for fetal liver macrophages, and the cells were cultured for only 10 days. At that point, they appeared to be essentially 100% macrophages, as

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determined by morphology and nonspecific esterase staining. On day 10, the cells were rinsed once with RPMI, 2 mM glutamine, P/S, 30 mM HEPES (pH 7.4), 0.04% (w/v) sodium bicarbonate and 10% FCS, and  
5 incubated in the same medium for 24 h. At that point, the medium was removed and replaced by fresh medium after one wash, and the cells were incubated in the presence or absence of 1  $\mu$ g/ml LPS for 4 hr. The supernatants were then harvested and assayed for TNF $\alpha$   
10 accumulation by western blot, as described below; the cells were harvested and used for the measurement of TNF $\alpha$  and glyceraldehyde-3-phosphate dehydroxogenase (GAPDH) mRNA levels, as described below.

*Preparation of B and T lymphocytes:* B and T  
15 lymphocytes were prepared from spleen and thymus of 11 day old TTP (+/+) and (-/-) littermate mice. The animals were euthanized by CO<sub>2</sub> inhalation, and spleen and thymus were aseptically removed. Tissues were placed in 10 ml of RPMI supplemented with 10% (v/v)  
20 FCS, P/S and 2 mM glutamine. Single cell suspensions from thymus were obtained according to the protocol described by Kruisbeek (Kruisbeek, A.M. In Current Protocols in Immunology., Coligan et al, editors. John Wiley and Sons, Inc., New York, NY (1995)). B  
25 lymphocytes were purified from spleens by complement mediated lysis of T cells, as described (Sato et al, Proc. Natl. Acad. Sci. USA 92:11558 (1995)), using the monoclonal antibodies H57-597 (anti-TCR) and 145-2C115 (anti-CD3) (Nishimura et al, Immunology 83:196  
30 (1994)) (provided by Dr. Thomas F. Tedder, Duke University, Durham, NC). B and T lymphocytes were cultured at 10<sup>6</sup> cells/ml in RPMI/10% FCS, in the presence of different agents [10 ng/ml of recombinant murine IL-2 (R&D Systems, Minneapolis, MN), 1  $\mu$ g/ml  
35 PHA (Sigma Chemical Co.) and 1  $\mu$ g/ml LPS for T



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lymphocytes; increasing concentrations of LPS for B lymphocytes (0, 1, 10, 100, 1000 ng/ml)] for 24 hr, and supernatants were harvested to measure the accumulation of TNF $\alpha$  by western blot, as described below.

Western blotting: Supernatants from cultured macrophages, and B and T lymphocytes, were removed, centrifuged at 15,000 rpm in an Eppendorf benchtop centrifuge for 10 min at 4°C to eliminate any floating cells, and mixed with 1/5 volume of 5X SDS-sample buffer (Blackshear, P.J., Methods Enzymol. 104:237 (1984)). Volumes of supernatant corresponding to  $1.25 \times 10^5$  cells were loaded onto 16% SDS-polyacrylamide gels (Protogel, National Diagnostics, Atlanta, GA), and subjected to electrophoresis at 40 V for 16 hr. Proteins were then transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) for 2 hr in 20% methanol, 150 mM glycine and 48 mM Tris-HCl (pH 8.3) (845 mMamp, room temperature), using a Hoeffer Transphor Electro-transfer Unit (Hoeffer, San Francisco, CA). After transfer of the proteins, the membranes were incubated in 7.5% (w/v) non-fat milk in Tris-buffered saline (TBS)/0.5% (v/v) Tween 20 at room temperature for 1 hr. The primary antibody against TNF $\alpha$ , a rabbit-anti-mouse antiserum (Kull et al, J. Cell. Biochem. 42:1 (1990)) (provided by Dr. Fred Kull (Glaxo Wellcome Inc., Research Triangle Park, NC) was diluted 1:2500 in TBS/0.5% Tween 20, and was incubated with the membranes for 1 hr at room temperature. The membranes were then washed in TBS/0.5% Tween 20 (2x5 min, 2x10 min) and then secondary antibody (goat-anti-rabbit, horseradish peroxidase-conjugated, Bio-Rad, Hercules, CA) was added at a 1:5000 dilution in the same buffer and

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incubated for a further 30 min at room temperature. The membranes were washed again four times as described above and developed with the Amersham ECL Detection System (Amersham Corporation, Arlington Heights, IL), as recommended by the manufacturer. After ECL, films were analyzed using a Zeineh Soft Laser Scanning Densitometer, model SL-504-XL (Biomed Instruments, Inc., Fullerton, CA).

*Northern blotting:* Total cellular RNA was obtained from marrow macrophage cultures and fetal liver-derived macrophages cultures using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH), according to the method described by Chomczynski (Chomczynski, P., BioTechniques 15:532 (1993)). Genomic DNA was isolated from the same samples and used to estimate the number of cells present in each sample. 10  $\mu$ g of RNA for the bone marrow-derived macrophages and 3.8  $\mu$ g from the fetal liver-derived macrophages were separated on 1.2% agarose/formaldehyde gels and processed for northern blotting as described (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)). The filters were successively probed with a mouse cDNA for TNF $\alpha$  (ATCC) and a rat cDNA for GAPDH (Tso et al, Nucl. Acid Res. 13:2485 (1985)). PhosphorImager analysis was used for quantitation of the blots; the results were expressed as quotients of TNF $\alpha$  mRNA  $\div$  GAPDH mRNA from each animal, and these quotients were then averaged and compared using Student's t test.

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### Results

RAG-2 (-/-) female mice at 7-9 weeks of age received bone marrow from either TTP (-/-) or (+/+) male mice. None of the recipient mice showed any acute adverse effects within one month of transplantation that could suggest graft versus host disease. Engraftment was assessed by the appearance of mature lymphocytes in peripheral blood, which occurred within one month of transplantation in all 10 recipients of both TTP (-/-) or (+/+) bone marrow. At various times during the study, recipient animals were euthanized and analyzed pathologically; at each point, a (-/-) marrow recipient was compared to a (+/+) marrow recipient.

Body weight: Loss of weight or failure to gain weight normally is one of the most prominent aspects of the TTP-deficiency phenotype (Taylor et al, Immunity 4:445 (1996)). No evident differences in the growth curves of the (+/+) and (-/-) recipients were apparent for the first 3 months after transplantation. However, three of the five recipients of (-/-) marrow then exhibited varying degrees of weight loss, ranging from profound (mouse #8) to moderate (#10 and #2). Of the recipients of (-/-) marrow, mouse #1 was selected randomly for pathological evaluation at 10 weeks after transplantation, before any of the animals showed evidence of weight loss, and mouse #7 never exhibited weight loss, despite repeated detection of circulating lymphocytes. None of the five recipients of (+/+) marrow exhibited weight loss or clinical or pathological abnormalities.

The weight loss was accompanied by nearly complete absence of adipose tissue in any of the

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- usual fat depots (subcutaneous, mesenteric, perirenal) in 3/5 of the mice receiving (-/-) marrow (see below). Even mouse #1, randomly selected for pathological examination 10 weeks after
- 5 transplantation, exhibited less adipose tissue than its control. Mouse #7 was the only recipient of TTP (-/-) marrow that appeared to have normal amounts of fat.

- Histology:* Histological evaluation of the
- 10 recipients of (-/-) marrow revealed evidence of both medullary and extramedullary myeloid hyperplasia, as described previously for the TTP-deficient mice (Taylor et al, Immunity 4:445 (1996)). The bone marrow from all of the (-/-) recipient mice appeared
- 15 grossly white, in contrast to the red marrow from the (+/+) recipients; this difference was consistently observed in previous study of the TTP (-/-) and (+/+) mice (Taylor et al, Immunity 4:445 (1996)). Marrow from all of the (-/-) recipients exhibited an
- 20 increase in myeloid cells compared to the (+/+) recipients [73.8  $\pm$  11.5% myeloid cells in the (-/-) recipients (n=5) versus 47.2  $\pm$  8% myeloid cells in the (+/+) recipients (mean  $\pm$  SD, n=5), p=0.007].

- Peripheral blood white cell counts were
- 25 increased only in mouse #2 [98x10<sup>3</sup>/ $\mu$ l compared to the control range of 5.7  $\pm$  2.1 x 10<sup>3</sup>/ $\mu$ l (n=2)]. Peripheral blood smears from this mouse showed an increase in circulating myeloid cells, both from the granulocytic and monocytic lineages [77.3% myeloid
- 30 cells (monocytes + granulocytes) in mouse #2, versus 52% myeloid cells in the age-matched controls].

- Livers from the (-/-) recipient mice appeared grossly normal at autopsy in four out of five animals. However, mouse #2 exhibited significant
- 35 enlargement of the liver [1.8 g, compared to 1.2  $\pm$

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0.3 g (n=2) in the age-matched controls]. Multiple white nodules were apparent on the surface of the organ; these consisted largely of granulocytes, which also infiltrated the rest of the hepatic parenchyma.

5     Imprints from these nodules confirmed the presence of myeloid cells at different stages of differentiation. The liver from mouse #7 also contained foci of granulocytes, with massive granulocytic infiltration of the gall bladder wall. Abnormal foci of

10    granulocytes were not detected in the livers of the other three (-/-) recipients, or in the livers of any of the (+/+) recipients.

Splenomegaly was not generally present in the (-/-) recipient mice, except for mouse #2, whose spleen

15    weighed 374 mg (compared to  $90.5 \pm 2.8$  mg in age-matched controls, n=2). However, all (-/-) recipients exhibited infiltration of the spleen with myeloid cells. They also contained increased numbers of megakaryocytes compared to the (+/+) recipient

20    spleens. Imprints of the spleens from the (-/-) recipients revealed myeloid cells in all stages of differentiation. In the (-/-) recipients, the increase in the number of myeloid cells was accompanied by destruction of the normal architecture

25    of the spleen, without the clear distinction between the white and red pulp.

Mouse #2 [(-/-) recipient] also exhibited lymphadenopathy, in which myeloid cells were also present.

30    The skin from the (+/+) recipients appeared normal. In 3 out of 5 (-/-) recipient mice, the skin completely lacked subcutaneous fat, as seen in TTP (-/-) mice (Taylor et al, Immunity 4:445 (1996)). Some animals exhibited a perivascular inflammatory

35    infiltrate in the dermis.

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The carpal joints from the (-/-) recipient mice showed varying extents of inflammatory pannus, bone erosion and bone destruction. As in the TTP-deficient mice (Taylor et al, Immunity 4:445 (1996)), there was generalized enlargement of the marrow cavities in all of the (-/-) recipients, which were full of active hematopoietic cells, particularly of the myeloid lineage. In contrast, the (+/+) recipients exhibited mostly fat and very few hematopoietic cells in the marrow. Neither mouse #1, which was analyzed only 10 weeks after marrow transplantation, nor mouse #7, which appeared to be healthy after 10 months of follow up, exhibited an inflammatory pannus in the carpal joints. However, mice #2, #8, and #10 exhibited varying amounts of inflammatory pannus that completely destroyed the bone surface and articular cartilage and infiltrated the marrow cavities.

**Autoantibodies:** Four months after marrow transplantation, only mouse #2 exhibited antinuclear antibodies and anti-DNA antibodies, both by ELISA and the Chrithidia assay. However, by the end of the study, all three (-/-) recipients studied were positive for antibodies to both single- and double-stranded DNA [for single-stranded DNA the mean  $\pm$  SD of ELISA units from the 3 (-/-) recipient mice was  $0.52 \pm 0.42$  compared with  $0.014 \pm 0.005$  for the two (+/+) recipients; for double-stranded DNA, the values were  $0.5 \pm 0.37$  for the (-/-) recipients versus  $0.034 \pm 0.026$  for the (+/+) recipients]. Anti-nuclear antibodies were positive in mouse #2, but negative in the other two (-/-) recipients and in both of the (+/+) recipients studied.

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*In situ hybridization for Y chromosome:* In situ hybridization with a DNA probe for mouse chromosome Y was performed in an attempt to establish the origin of the myeloid cells that were present in the transplanted animals. In bone marrow there was a mixed population of myeloid cells positive for the Y chromosome (from the donor) and cells that were negative (from the recipient) in both (-/-) and (+/+) marrow recipients. In mouse #2, in situ hybridization was also performed on sections of spleen, lymph nodes and liver. Virtually the entire spleen and lymph nodes showed an intense hybridization signal, mainly due to the presence of mature lymphocytes originating from the donor. In the liver, only granulocytes and myeloid cells were positive for the Y chromosome, whereas the hepatocytes (recipient) were completely negative. However, the number of Y chromosome positive granulocytes in the liver represented only about 50% of the total granulocytes present, as determined by hematoxylin-eosin staining.

*TNF $\alpha$  production:* Western blot studies were performed on supernatants from cultured macrophages and B and T lymphocytes in order to assess the production of TNF $\alpha$  by these cells. Three different sources of macrophages were used: resident peritoneal macrophages, obtained by lavage of the peritoneal cavity of adult mice; fetal liver-derived macrophages, obtained by culturing the hematopoietic precursors present in the fetal liver between days 14-16 of gestation; and bone marrow-derived macrophages, obtained by culturing the hematopoietic precursors from the bone marrow of adult mice.

In all three cases, macrophages obtained from TTP (-/-) animals showed an increased accumulation of

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TNF $\alpha$  in the medium when stimulated with LPS. Thus, peritoneal macrophages from TTP (-/-) mice, incubated for 24 hr in the presence of 10 ng/ml of LPS, secreted readily detectable amounts of TNF $\alpha$  into the culture medium. By contrast, under the same conditions, peritoneal macrophages from TTP (+/+) mice did not produce detectable amounts of TNF $\alpha$  under western blot conditions used. In these mice, measurable TNF $\alpha$  accumulation only occurred after 24 hr of incubation in the presence of 100 ng/ml LPS, and even then, the levels of TNF $\alpha$  were much lower than those observed for the macrophages from the TTP (-/-) mice. Densitometry of the autoradiographs indicated that there was a 5.6-fold increase in the levels of TNF $\alpha$  present in the supernatants from the TTP (-/-) peritoneal macrophages compared to the TTP (+/+) cells exposed to 100 ng/ml LPS.

Macrophages derived from fetal liver exhibited essentially the same behavior. TNF $\alpha$  was readily detectable in the culture supernatants after 24 hr in the presence of 1 ng/ml LPS, but at much higher levels in the (-/-) macrophages than in the (+/+) ones. These autoradiographs were analyzed by densitometry, and the results compared using Student's t test. At all LPS concentrations studied, there was about a five-fold greater accumulation of TNF $\alpha$  in the supernatants from the (-/-) cells compared to the (+/+) cells; these differences were statistically significant ( $p < 0.05$ ). Longer exposure of these blots showed that, even in the absence of LPS, there was greater TNF $\alpha$  accumulation in the supernatants from the cells compared to the (+/+) cells.

Bone marrow-derived macrophages were exposed to 1  $\mu$ g/ml LPS for 4 hr, and the levels of TNF $\alpha$  present in the culture supernatants were assayed by western



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blot. In all cases, the media from the (-/-) macrophages contained much higher levels of TNF $\alpha$  than the media from the cells. Densitometry of these autoradiographs revealed that there was a seven-fold greater accumulation of TNF $\alpha$  in the supernatants from the (-/-) cells compared to the (+/+) cells; this difference was statistically significant ( $p < 0.01$ ).

Analysis of TNF $\alpha$  production by B and T lymphocytes obtained from TTP (-/-) and mice did not reveal any significant differences between cells of the two genotypes. TNF $\alpha$  mRNA levels were studied in fetal liver-derived macrophages and in bone marrow-derived macrophages. In bone marrow-derived macrophages, the basal levels of TNF $\alpha$  mRNA were similar in (-/-) and (+/+) cells when normalized for GAPDH mRNA levels. However, after LPS stimulation (1  $\mu$ g/ml for 4 hr), the TNF $\alpha$  mRNA levels in (-/-) cells were approximately twice as great as the ones from the (+/+) cells ( $p < 0.05$ ). A similar two-fold difference was observed in the fetal liver-derived macrophages.

**Conclusions:** The above-described studies indicate that macrophage progenitors are among the transplanted cells that are capable of reconstituting the TTP-deficiency phenotype in RAG-2 (-/-) mice. These studies also indicate that macrophages are among the sources of TNF $\alpha$  overproduction in the TTP (-/-) animals.

\* \* \*

All documents cited above are hereby incorporated in their entirety by reference.

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One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

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WHAT IS CLAIMED IS:

1. A method of inhibiting tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) production, processing or secretion in a mammal comprising increasing the level of tristetraprolin (TTP), or a TNF $\alpha$  production, processing or secretion-inhibitory polypeptide fragment thereof, in said mammal so that said inhibition is effected.

2. The method according to claim 1 wherein said level of TTP is increased by administering to said mammal a nucleic acid sequence encoding TTP, or said polypeptide fragment thereof, and effecting expression of said sequence so that said level of TTP, or polypeptide fragment thereof, is increased.

3. The method according to claim 2 wherein said nucleic acid sequence encodes TTP.

4. The method according to claim 2 wherein said nucleic acid sequence is present as an insert in an expression vector.

5. The method according to claim 4 wherein said vector is a viral vector.

6. The method according to claim 2 wherein said nucleic acid sequence is present in a liposome.

7. A method of treating an effect of excess TNF $\alpha$  in a mammal comprising administering to said mammal TTP, or polypeptide fragment thereof that inhibits TNF $\alpha$  production, processing or secretion, or agent that enhances a TNF $\alpha$  production, processing or

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secretion-inhibitory effect of TTP, in an amount sufficient to effect treatment.

8. The method according to claim 7 wherein said TTP, or said polypeptide fragment thereof, is administered by introducing into said mammal a nucleic acid sequence encoding said TTP, or said polypeptide fragment thereof, and effecting expression of said sequence.

9. The method according to claim 7 wherein said effect of said excess is inflammation, infection or cancer cachexia.

10. The method according to claim 7 wherein said effect is rheumatoid arthritis, Type I diabetes or systemic lupus erythematosus.

11. A method of identifying a subject susceptible to a  $TNF\alpha$  associated disease or disorder comprising isolating a DNA-containing biological sample from said subject, locating the TTP gene present in said DNA and comparing the nucleic sequence of said TTP gene with a wild-type TTP encoding sequence and thereby determining whether said TTP gene includes a mutation that renders said subject susceptible to said disease or disorder.

12. The method according to claim 11 wherein, after locating the TTP gene, at least one polymorphic locus linked to said TTP gene is identified.

13. A method of identifying a subject susceptible to a  $TNF\alpha$  associated disease or disorder comprising

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- i) isolating a biological sample from said subject,
- ii) contacting said sample with a TTP binding partner under conditions such that complexation between TTP and said binding partner can occur, and
- iii) detecting the presence or absence of said complexation, or comparing the extent of said complexation with a control sample comprising wild-type TTP.

14. The method according to claim 13 wherein said binding partner is an anti wild-type TTP antibody.

15. A method of screening a compound for its ability to enhance the ability of TTP to inhibit TNF $\alpha$  production comprising

- i) contacting said compound with a sample comprising a TNF $\alpha$  encoding sequence, in the presence of TTP or TNF $\alpha$  production-inhibitory polypeptide fragment thereof, under conditions such that said TNF $\alpha$  encoding sequence can be expressed, and
- ii) determining the level of expression of said TNF $\alpha$  encoding sequence and comparing that level to a level of expression obtained in the absence of said compound.

16. A method of screening a compound for its ability to enhance a TNF $\alpha$  transcription-repressor effect of TTP comprising

- i) contacting said compound with a sample comprising a TNF $\alpha$  promoter sequence operably linked to an encoding sequence, in the presence of TTP or TNF $\alpha$  production-inhibitory polypeptide fragment thereof, under conditions such that said encoding sequence can be expressed, and

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ii) comparing the level of expression of said encoding sequence obtained to a level of expression obtained in the absence of said compound.

17. The method according to claim 16 wherein said encoding sequence encodes a reporter protein.

18. The method according to claim 16 wherein said encoding sequence encodes TNF $\alpha$ .

19. A method of screening a compound for its ability to enhance a TNF $\alpha$  mRNA translation-inhibitory effect of TTP comprising

i) contacting said compound with a sample comprising TNF $\alpha$  mRNA, in the presence of TTP or a TNF $\alpha$  translation-inhibitory fragment thereof, under conditions such that translation of said TNF $\alpha$  mRNA can be effected, and

ii) determining the level of translation of said TNF $\alpha$  mRNA and comparing that level of translation of said TNF $\alpha$  mRNA to a level of translation of TNF $\alpha$  mRNA obtained in the absence of said compound.

20. A method of screening a compound for its ability to enhance the ability of TTP to inhibit TNF $\alpha$  processing comprising

i) contacting the compound with a sample comprising TNF $\alpha$  and TTP or TNF $\alpha$  processing-inhibitory polypeptide fragment thereof, and

ii) determining the level of processed TNF $\alpha$  in said sample and comparing that level to a level obtained in the absence of said compound.

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21. A method of screening a compound for its ability to enhance the ability of TTP to inhibit TNF $\alpha$  secretion from a cell comprising

- i) contacting a cell comprising TNF $\alpha$  and TTP or a TNF $\alpha$  secretion-inhibitory polypeptide fragment thereof, with said compound, and
- ii) determining the amount of TNF $\alpha$  secreted from said cell and comparing that amount to an amount obtained in the absence of said compound.

22. A TTP-deficient non-human mammal.

23. The mammal according to claim 22 wherein said mammal is a rodent.

24. The mammal according to claim 22 wherein a TTP gene present in the genome of said mammal is disrupted.

25. A method of screening or testing a compound for its ability to treat a symptom of excess TNF $\alpha$  comprising administering said compound to said mammal according to claim 22 and monitoring the effect of said compound on said symptom.

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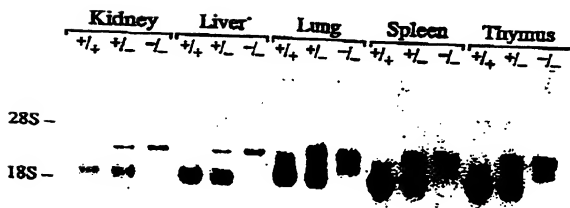
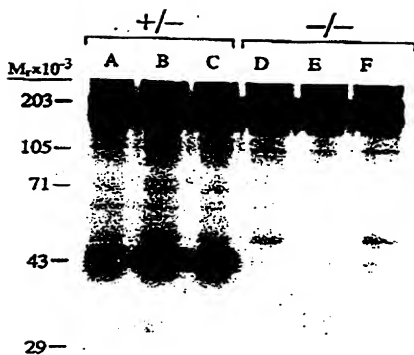


FIG 1A



2 / 5

Fig 13

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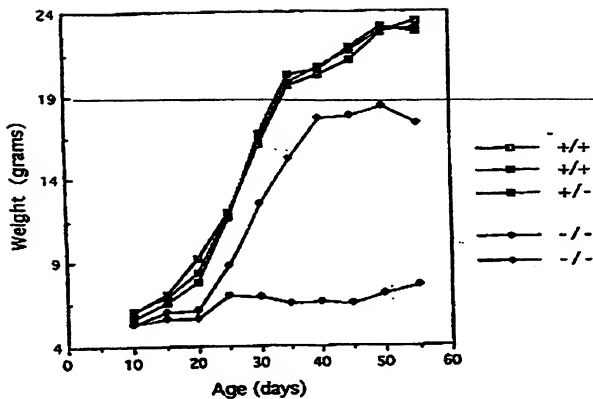
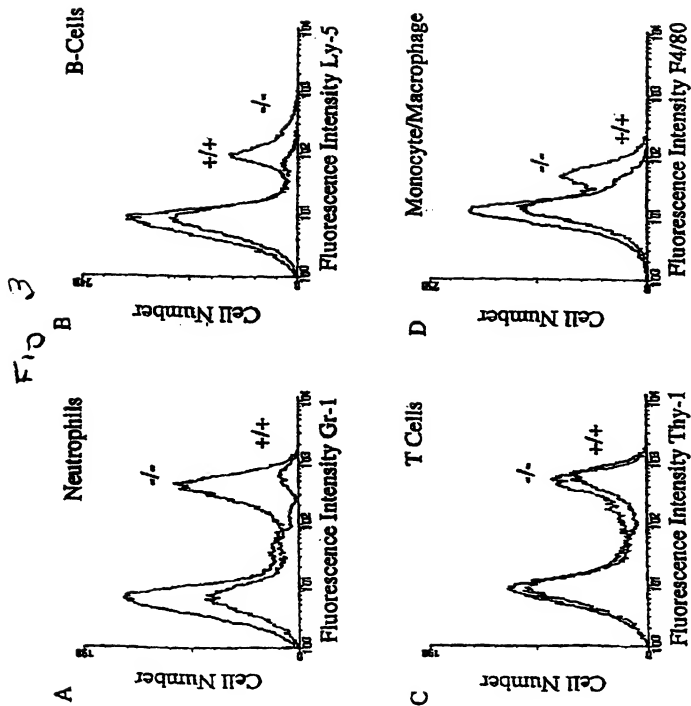


Fig. 2



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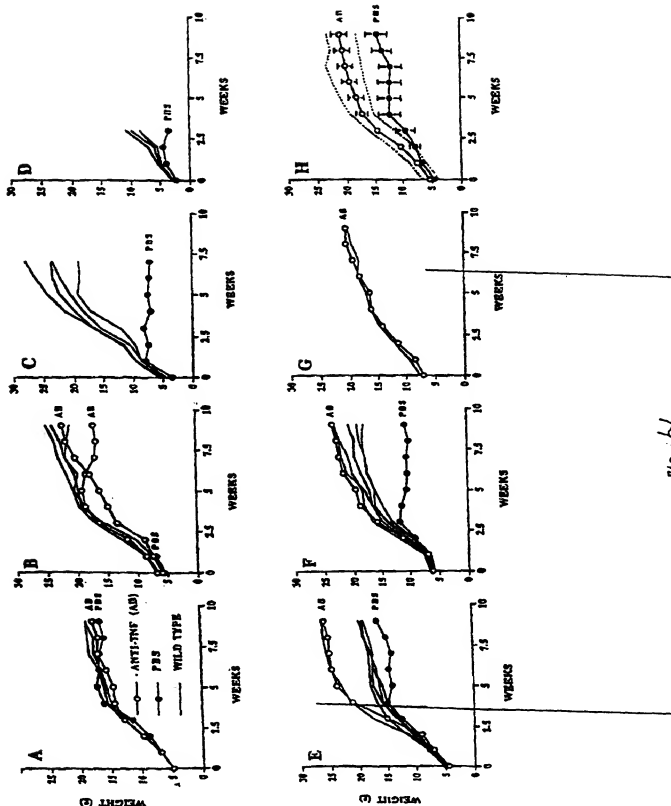


Fig. 4

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/08394

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A01N 43/04; C12Q 1/68; G01N 33/566

US CL : 514/2, 44; 435/6; 436/501; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 44; 435/6, 235.1, 320.1; 436/501; 800/2; 514/2; 530/350; 536/23.1, 23.5, 24.31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,E	US 5,641,751 A (HEAVNER) 24 June 1997, see entire document.	7-10, 15-18, 25
Y,E	US 5,650,156 A (GRINSTAFF et al.) 22 July 1997, see entire document.	1-6
Y,E	US 5,648,251 A (KOTANI et al.) 15 July 1997, see entire document.	1-6
Y,E	US 5,646,154 A (IRIE et al.) 08 July 1997, see entire document.	7-10, 19-21
Y,E	US 5,641,680 A (ZHAO) 24 June 1997, see entire document.	1-6
Y,E	US 5,635,380 A (NAFTILAN et al.) 03 June 1997, see entire document.	1-6

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	T* later documents published after the international filing date or priority date and not in conflict with the application but cited to understate the principle or theory underlying the invention
A* document defining the general state of the art which is not considered to be of particular relevance	X* documents of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E* earlier document published on or after the international filing date	Y* documents of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	A* documents member of the same patent family
O* document referring to an oral disclosure, use, exhibition or other means	
P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search	Date of mailing of the international search report
24 JULY 1997	29 AUG 1997

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/08394

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,580,859 A (FELGNER et al.) 03 December 1996, see entire document.	1-6
Y,P	US 5,576,206 A (SCHLEGEL) 19 November 1996, see entire document.	1-6
Y,P	US 5,567,433 A (COLLINS) 22 October 1996, see entire document.	1, 2, 6-10
Y,P	US 5,547,970 A (WEITHMANN et al.) 20 August 1996, see entire document.	7-10, 15-21, 25
Y,P	US 5,519,000 A (HEAVNER et al.) 21 May 1996, see entire document.	7-10, 15-18, 25
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<b>(54) Title:</b> TRISTETRAPROLIN			
<b>(57) Abstract</b>  The present invention relates, in general, to tristetraprolin (TTP) and, in particular, to methods of modulating levels of tumor necrosis factor $\alpha$ (TNF $\alpha$ ) using TTP or nucleic acid sequences encoding same. The invention further relates to methods of screening for compounds for their ability to inhibit TNF $\alpha$ biosynthesis.			

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**TRISTETRAPROLIN**

This is a continuation-in-part of Application No. 08/648,773, filed May 16, 1996, the entire contents of which are hereby incorporated by reference.

This application was made with Government support under Grant No. \_\_\_\_\_ awarded by the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

The present invention relates, in general, to tristetraprolin (TTP) and, in particular, to methods of modulating levels of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) using TTP or nucleic acid sequences encoding same. The invention further relates to methods of screening compounds for their ability to inhibit TNF $\alpha$  biosynthesis, processing or secretion.

BACKGROUND

Tumor necrosis factor alpha (TNF $\alpha$ ) is a potent cytokine that is released from many cell types, particularly, macrophages and monocytes. TNF $\alpha$  also exists in a cell-membrane bound, higher molecular weight form on cells, and this form also appears to mediate a variety of biological effects. TNF $\alpha$  is thought to have few roles in normal development and physiology; however, it exerts harmful and destructive effects on many tissues in many disease states (Tracey et al, Ann. Rev. Med. 45:491 (1994)). Disease states in which TNF $\alpha$  has been shown to exert a major pathogenetic role include septic shock syndrome, cancer cachexia, rheumatoid arthritis, etc.

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Many investigators and pharmaceutical companies are actively investigating agents and potential drugs that can block TNF $\alpha$  effects, either by blocking its synthesis or interfering with its binding to its surface receptors.

One example of this approach is the use of monoclonal antibodies to TNF $\alpha$ . These have been used in animal models of human disease, and in human conditions such as rheumatoid arthritis (Arend et al, Arthritis and Rheumatism 38:151 (1995)). There is no question that these antibodies temporarily relieve some of the signs and symptoms of this disease in man. However, their potential widespread use is compromised by many factors, especially the fact that they seem to be only temporarily (ie, a few months) effective. Other drawbacks include expense, the need for parenteral administration, the likelihood that anti-idotype antibodies will develop, etc.

The present invention relates to a novel approach to the treatment of diseases the effects of which are mediated, at least in part, through TNF $\alpha$ . This approach involves the protein tristetraprolin (TTP) and nucleic acid sequences encoding same.

TTP (Lai et al, J. Biol. Chem. 265:16556 (1990)), also known as Nup475 (DuBois et al, J. Biol. Chem. 265:19185 (1990)) and TIS11 (Varnum et al, Oncogene 4:119 (1989); Varnum et al, Mol. Cell. Biol. 11:1754 (1991)), is a widely distributed 33.6 kDa phosphoprotein encoded by the immediate-early response gene, Zfp-36 (Taylor et al, Nucl. Acids Res. 19:3454 (1991)). This gene has been mapped to chromosome 7 in the mouse, and the equivalent human gene, ZFP36, has been mapped to chromosome 19q 13.1 (Taylor et al, Nucl. Acids Res. 19:3454 (1991)). TTP is the prototype of a group of proteins containing two or more highly conserved putative zinc fingers of

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the CCH class (Varnum et al, Mol. Cell. Biol. 11:1754 (1991); Taylor et al, Nucleic Acids Res. 19:3454 (1991); Gomperts et al, Oncogene 5:1081 (1990); Ma et al, Oncogene 9:3329 (1994)). In addition, the protein has been shown to bind Zn<sup>2+</sup> and has been localized to the cell nucleus in mouse fibroblasts (DuBois et al, J. Biol. Chem. 265:19185 (1990)), suggesting that it may be a transcription factor. Serum or other mitogen stimulation of quiescent fibroblasts causes rapid serine phosphorylation and nuclear to cytosolic translocation of TTP (Taylor et al, J. Biol. Chem. 270:13341 (1995); Taylor et al, Mol. Endocrinol. 10:140 (1996)), both of which are likely to modulate its function in cells.

In the adult mouse, TTP mRNA is highly expressed in lung, intestine, lymph node, spleen, and thymus, with lower expression in adipose tissue, kidney, and liver, and negligible expression in skeletal muscle and brain (Lai et al, J. Biol. Chem. 265:16556 (1990); DuBois et al, J. Biol. Chem. 265:19185 (1990)). In the thymus, TTP mRNA is highly expressed in both cortical and medullary thymocytes, while in the spleen, it is highly expressed in B and T lymphocytes within the white pulp, and is expressed at somewhat lower levels in the myeloid cells of the red pulp and endothelial cells of the high endothelial venules. In addition, TTP is constitutively expressed in several types of blood cells, particularly neutrophils, macrophages and B and T lymphocytes. The function of TTP in normal vertebrate physiology, however, was heretofore unknown.

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OBJECTS AND SUMMARY OF THE INVENTION

It is a general object of the invention to provide a method of modulating cellular levels of TNF $\alpha$ .

5 It is a specific object of the invention to provide a method of treating diseases and disorders associated with TNF $\alpha$  excess.

It is a further object of the invention to provide a method of identifying an individual at  
10 increased risk to the effects of TNF $\alpha$  excess.

It is another object of the invention to provide a method of selecting compounds for there ability to inhibit TNF $\alpha$  production, processing or secretion.

15 It is a further object of the invention to provide a TTP-deficient non-human mammal.

These objects are met by the present invention.

In one embodiment, the present invention relates to a method of inhibiting TNF $\alpha$  production, processing or secretion in a mammal. The method comprises  
20 increasing the level of TTP, or a TNF $\alpha$  production, processing or secretion-inhibitory polypeptide fragment thereof, in the mammal so that the inhibition is effected.

In a further embodiment, the present invention  
25 relates to a method of treating an effect of excess TNF $\alpha$  in a mammal. The method comprises administering to the mammal TTP, or polypeptide fragment thereof that inhibits TNF $\alpha$  production, processing or secretion, or agent that enhances a TNF $\alpha$  production,  
30 processing or secretion-inhibitory effect of TTP, in an amount sufficient to effect the treatment.

In yet another embodiment, the present invention relates to a method of identifying a subject  
susceptible to a TNF $\alpha$  associated disease or disorder.  
35 The method comprises isolating a DNA-containing

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biological sample from the subject, locating the TTP gene present in the DNA and comparing the nucleic sequence of the TTP gene with a wild-type TTP encoding sequence and thereby determining whether the TTP gene includes a mutation that renders the subject susceptible to the disease or disorder.

In a further embodiment, the present invention relates to a method of identifying a subject susceptible to a TNF $\alpha$  associated disease or disorder. The method comprises

- i) isolating a biological sample from the subject,
- ii) contacting the sample with a TTP binding partner under conditions such that complexation between TTP and the binding partner can occur, and
- iii) detecting the presence or absence of the complexation, or comparing the extent of the complexation with a control sample comprising wild-type TTP.

In another embodiment, the present invention relates to a method of screening a compound for its ability to enhance the ability of TTP to inhibit TNF $\alpha$  production. The method comprises

- i) contacting the compound with a sample comprising an expression construct comprising a TNF $\alpha$  encoding sequence, in the presence of TTP or TNF $\alpha$  production-inhibitory polypeptide fragment thereof, under conditions such that the TNF $\alpha$  encoding sequence can be expressed, and
- ii) determining the level of expression of the TNF $\alpha$  encoding sequence and comparing that level to a level of expression obtained in the absence of the compound.

In yet a further embodiment, the present invention relates to a method of screening a compound

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for its ability to enhance a TNF $\alpha$  transcription-repressor effect of TTP. The method comprises

- 5 i) contacting the compound with a sample comprising an expression construct comprising a TNF $\alpha$  promoter sequence operably linked to an encoding sequence, in the presence of TTP or TNF $\alpha$  production-inhibitory polypeptide fragment thereof, under conditions such that the encoding sequence can be expressed, and
- 10 ii) comparing the level of expression of the encoding sequence obtained to a level of expression obtained in the absence of the compound.

In another embodiment, the present invention relates to a method of screening a compound for its ability to enhance a TNF $\alpha$  mRNA translation-inhibitory effect of TTP. The method comprises

- 15 i) contacting the compound with a sample comprising TNF $\alpha$  mRNA, in the presence of TTP or a TNF $\alpha$  translation-inhibitory fragment thereof, under conditions such that translation of the TNF $\alpha$  mRNA can be effected, and
- 20 ii) determining the level of translation of the TNF $\alpha$  mRNA and comparing that level of translation of the TNF $\alpha$  mRNA to a level of translation of TNF $\alpha$  mRNA obtained in the absence of the compound.

25 In yet a further embodiment, the present invention relates to a TTP-deficient non-human mammal.

In another embodiment, the present invention  
30 relates to a method of screening or testing a compound for its ability to treat a symptom of excess TNF $\alpha$ . The method comprises administering the compound to a TTP-deficient non-human mammal and monitoring the effect of the compound on the symptom.



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Further objects and advantages of the invention will be clear from the description that follows.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Expression of TTP mRNA (a) and protein (B). (a) Total RNA was isolated from the indicated tissues of (+/+), (+/-), and (-/-) mice and subjected to northern blotting with a TTP cDNA probe. Each lane contains 15 $\mu$ g of total cellular RNA. The lower band represents endogenous TTP mRNA, whereas the upper band represents the TTP-neo fusion RNA. (b) Primary embryonic fibroblasts were isolated from three different (+/-) (A-C) and three (-/-) (D-F) embryos. The cells were serum-deprived for 14 h, and then exposed to [<sup>35</sup>S]-cysteine for 2 h and 20% fetal calf serum for an additional 2 h. Lysates from these cells were used for immunoprecipitation with an antibody that recognizes the amino-terminus of TTP, and the immunoprecipitated proteins were separated on a 9% acrylamide SDS gel and an autoradiograph prepared. The positions of molecular weight standards are indicated. TTP migrates at about 43 kDa. Immunoprecipitated proteins from the same samples were also separated on a 20% acrylamide SDS gel; no truncated amino-terminal TTP fragment was detected in the (-/-) samples.

Figure 2. Growth curves of two -/- and three +/+ or +/- littermates. Weekly weights of one litter of five pups were determined; genotypes of each mouse are indicated to the right.

Figure 3. Flow cytometric analysis of (a) Gr-1<sup>+</sup> neutrophils, (b) Ly-5<sup>+</sup> lymphocytes, (c) Thy-1<sup>+</sup> T lymphocytes, and (d) F4/80<sup>+</sup> monocytes/macrophages, in

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peripheral blood adult (+/+) and (-/-) mice. Cell surface analysis was performed using a Becton Dickinson FACStar Plus flow cytometer and the accompanying software. Shown are the results from representative mice. The average percent positive cells and average absolute number of positive cells per mm<sup>3</sup> ( $\pm$  SEM) from 5-7 adult mice were: Gr-1: (+/+) 23 $\pm$ 4%, 1.13 $\pm$ 0.27X10<sup>3</sup>; (-/-) 48 $\pm$ 2%, 4.36 $\pm$ 0.72x10<sup>3</sup>; Ly-5: (+/+) 26 $\pm$ 3%, 1.36 $\pm$ 0.28X10<sup>3</sup>; (-/-) 6 $\pm$ 1%, 0.71 $\pm$ 0.15x10<sup>3</sup>; Thy-1: (+/+) 33 $\pm$ 6%, 1.67 $\pm$ 0.53x10<sup>3</sup>; (-/-) 33 $\pm$ 3%, 3.41 $\pm$ 0.39x10<sup>3</sup>; F4/80: (+/+) 5 $\pm$ 1%, 0.29 $\pm$ 0.05x10<sup>3</sup>; (-/-) 8 $\pm$ 2%, 0.87 $\pm$ 0.26x10<sup>3</sup>. Similar analyses from spleen and bone marrow are discussed in the text.

Figure 4. Effect of TNF $\alpha$  antibody injections on body weights. As described in the text, TTP -/- mice from seven litters (each litter is labeled A-G) were injected at weekly intervals starting on day 10 of age with either anti-TNF monoclonal antibodies (AB; open circles) or PBS (closed circles). Body weights were measured at weekly intervals and are shown here. Littermates of the TTP +/+ and +/- genotypes are indicated by the lines without symbols. TTP -/- mice from litters B, C and D that were injected with PBS died before completion of the experiments, which consisted of nine weekly injections. The means  $\pm$  SEM of the six antibody-injected animals and the six PBS-injected animals are shown in H; the normal range (mean  $\pm$  S.D.) from all of the control littermates (n = 25) is indicated by the dashed lines in H. The differences between the AB and PBS means were significant (p < 0.001 using Student's t test) at each time point after four weeks.

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DESCRIPTION OF THE INVENTION

5       The present invention results from the realization that TTP regulates effective levels of TNF $\alpha$  in animals, eg mammals. As indicated above, TNF $\alpha$  has been implicated in the pathology of a variety of neoplastic diseases, immune disorders and infections. Accordingly, the ability of TTP to regulate TNF $\alpha$  levels is of considerable pharmaceutical importance.

10       The identification of this regulatory activity of TTP makes possible methods of screening compounds for their ability to enhance the TNF $\alpha$  production-inhibitory activity of TTP, as well as their ability to enhance the effect of TTP on TNF $\alpha$  processing and  
15       secretion. It also makes possible methods of identifying susceptibility to TNF $\alpha$ -associated diseases, including inflammatory conditions and sepsis. Further, the demonstration of TTP as an inhibitor of TNF $\alpha$  production, processing or secretion  
20       makes possible new modes of therapy for diseases or disorders mediated by or exacerbated by TNF $\alpha$ .

      It will be appreciated that the preferred subject of the invention is a human, however veterinary uses are also contemplated.

25       Compound Screens

      The natural ability of TTP to inhibit cellular production, processing or secretion of TNF $\alpha$  permits the screening of test compounds for their ability to enhance this inhibitory effect. Using appropriate  
30       screens, compounds can be identified that potentiate the ability of TTP to inhibit TNF $\alpha$  production, processing or secretion, independent of the mechanism by which this inhibition occurs.

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In one example of a screening system of the invention, a host cell is cotransfected with a vector encoding TTP and a vector containing the TNF $\alpha$  gene, for example, operably linked to a reporter gene.

5 Examples of commonly used reporter genes include chloramphenicol acyltransferase (CAT), alkaline phosphatase, luciferase, growth hormone, thymidine kinase, etc. Potential host cells include fibroblasts, HeLa cells, and macrophage and

10 lymphocyte cells lines. Transfection can be effected using art-recognized techniques.

Using this type of system, test compounds can be assayed for their ability to shift the TTP dose response curve to the left, that is, to decrease the

15 dose at which TTP inhibits TNF $\alpha$  production (or processing or secretion) as measured, for example, by the reporter expression. Compounds identified as being capable of enhancing (directly or indirectly) the TTP inhibitory activity can then be further

20 assayed, using standard protocols, for stability, toxicity etc.

While the foregoing screen can be used to assay test compounds for their ability to decrease the dose at which TTP inhibits TNF $\alpha$  production (or processing

25 or secretion), various other screens can be devised based on the mechanism by which TTP exerts its inhibitory effect. For example, if TTP inhibits TNF $\alpha$  gene transcription, then a screen can be used in which sequences within the TNF $\alpha$  gene promoter are

30 linked to a reporter gene. Such constructs can be used in cell transcription studies or cell-free transcription assays in the presence or absence of TTP and the test compound. Similarly, if TTP inhibits TNF $\alpha$  mRNA translation, test compounds can be

35 added, for example, to cell-free translation assays in the presence or absence of TTP and TNF $\alpha$  mRNA. The

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rate of synthesis of the TNF $\alpha$  protein can then be determined. Other screens can also be used, dependent on the biochemical site of action of TTP (eg gene transcription, mRNA translation, protein processing or protein secretion).

5           Examples I-V below describe an animal model of TTP deficiency produced using gene targeting in murine embryonic stem cells. The resulting mice, while appearing normal at birth, develop a phenotype  
10           consistent with whole body TNF $\alpha$  excess. This animal model (as well as other such TTP-deficient animal (eg mammalian) models) can be used to screen and/or test agents for their ability to prevent/treat the effects of excess TNF $\alpha$ . The test agent can be administered  
15           to the model animal (eg orally or by injection) in accordance with standard test protocols and the effects on animal growth and phenotype monitored. Compounds that prevent the development of one or more aspects of the phenotype of TTP-deficient animals can  
20           then be further tested for pharmaceutical acceptability using standard protocols.

#### Detection/Diagnosis

          The TTP deficiency produced in Examples I-V causes a severe syndrome of wasting and arthritis  
25           that is often lethal. Partial deficiencies resulting from the production of mutant forms of TTP, while perhaps non-lethal, can be expected to increase susceptibility to TNF $\alpha$  associated diseases and disorders, including infections, and autoimmune  
30           disorders (likewise, heterozygosity). The availability of the amino acid sequence of TTP (eg the human TTP sequence, Taylor et al, Nucl. Acids Res. 19:3454 (1991)) and its encoding sequence (eg GenBank accession number M63625) makes possible  
35           methods of identifying and diagnosing individuals at

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increased risk, for example, for inflammatory conditions or sepsis following infection.

General mutation screening of the TTP gene to identify subjects at increased risk can be performed by such methods as direct sequencing of cDNAs from candidate patients. Alternatively, approaches based on the cytogenetic mapping of the human gene to chromosome 19 q 13.1 (Taylor et al, Nucl. Acids Res. 19:3454 (1991)) can be used. For example, sequences from the human cDNA or genomic DNA can be used to locate the TTP gene (ZFP36) on a physical map of chromosome 19 (see, for example, Garcia et al, Genomics 27:52 (1995)). One or more polymorphic loci in the immediate vicinity of the gene can then be identified. Polymerase chain reaction (PCR) primers, for example, can be used to screen genomic DNA from populations (eg members of multiple families with rheumatoid arthritis) for polymorphisms closely linked to the TTP gene. Direct DNA sequencing of genomic DNA from likely candidate patients can then be accomplished using, for example, PCR sequencing strategies.

In an alternative approach, a biological sample can be obtained from a subject suspected of being at increased risk and the sample examined for the presence of a mutated form of TPP. Biological samples suitable for use in this regard include blood cells and transformed cell lines derived therefrom, lung lavage fluid, ascites fluid, etc. Tissue samples can also be used, including samples from liver, kidney intestine, spleen, lymph nodes, etc. Detection of a mutant form of the protein can be effected by isolating the protein from the sample and determining its amino acid sequence or by contacting the protein (in purified or semi-purified form) with a binding partner (eg an anti-wild type TTP antibody

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or TNF $\alpha$  promoter sequence) and determining whether complexation occurs. Complexation (or lack thereof) can be established using any of a variety of art recognized techniques (eg use of a labeled binding partner, use of a binding partner bound to a solid support, etc). Mutant forms of the protein can be expected to have altered (eg decreased) affinity for the binding partner. In addition, abnormalities in the size, charge or relative amount of the protein identified, for example, by means of a binding partner (such as an antibody) indicate a mutation in the TTP gene. Mutations can be confirmed by routine sequencing of the gene.

The identification of mutant forms TTP or its encoding sequence makes possible the identification of subjects likely to benefit from increased monitoring or therapeutic intervention.

#### Therapy

The present invention contemplates the use in gene therapy regimens of DNA sequences encoding TTP or portions thereof encoding TNF $\alpha$  production (processing or secretion)-inhibiting polypeptides. The encoding sequences can be present in a construct which, when introduced into target cells, results in expression of the TTP encoding sequences and thus production of the TNF $\alpha$  production (processing or secretion)-inhibitor.

For gene therapy to be practical, it is desirable to employ a DNA transfer method that: (1) directs the therapeutic sequence into specific target cell types (2) is highly efficient in mediating uptake of the therapeutic polynucleotide into the target cell population, and (3) is suited for use *ex vivo* or *in vivo* for therapeutic application.

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Delivery of the TTP gene (or portion thereof encoding a TNF $\alpha$  production (processing or secretion)-inhibitor polypeptide) can be effected using any of a variety of methodologies. Presently available  
5 methodologies include transfection with a viral vector (eg retroviral vector or adenoviral vector) and fusion with a lipid. Other techniques are also available, many employing selectable markers to improve transfection efficiency. The technique  
10 selected depends upon the particular situation.

Retroviral vectors can be used to effect high efficiency gene transfer into replicating cells and such vectors are particularly suitable for use where  
15 target cells are present in a body compartment, such as brain and liver or epithelial surfaces such as lung, bladder or colon. Adenovirus vectors are advantageous from the standpoint that they have the potential to carry larger insert polynucleotide sequences than retroviral vectors and they have the  
20 ability to infect non-replicating cells. Further, they are suitable for infecting tissues *in situ*, especially in the lung. Adenoassociated viruses, which integrate, can also be used, as can other viral systems depending on the target site, including  
25 hepatitis virus when liver is the target tissue. Consistent with this approach, TTP sequences can be transfected into autologous bone marrow progenitor or stem cells, and those cells can then be transplanted back into the original donor, for example, after  
30 selection for cells expressing the transfected TTP sequences. Similar approaches are contemplated in the treatment of rheumatoid arthritis (Chernajorsky et al, Brit. Med. Bull. 51:503 (1995); Kiem et al, Curr. Opin. Oncol. 7:107 (1995)). (See also Morgan



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and Anderson, Annu. Rev. Biochem. 62:191 (1993) and Mulligan, Science 260:926 (1993) for further details relating to use of viral vectors for gene therapy.)

Another gene transfer method suitable for use in the present invention is the physical transfer of plasmid DNA in liposomes directly into target cells. Liposome-mediated DNA transfer has been described by various investigators (Liu et al, Gene Therapy 1:7 (1994); Huxley, Gene Therapy 1:7 (1994); Miller and Vile, FASEB J. 9:190 (1995)).

Essentially any suitable DNA delivery method can be used in the context of the present invention. Ex vivo transfection using viral vectors, however, may be preferred in certain settings. Use of the TTP gene truncated at the 3' untranslated region may serve to make the mRNA more stable. Alternatively, a TTP cDNA operably linked to a cell specific promoter can be used. In any case, transfection of hematopoietic marrow progenitors or stem cells ex vivo and reintroduction by bone marrow transplantation can be effected.

The nucleic acid-containing compositions of the invention can be stored and administered in a sterile physiologically acceptable carrier. The nucleic acid can be present in combination with any agent which aids in the introduction of the DNA into cells.

Various sterile solutions may be used for administration of the composition, including water, PBS, etc. The concentration of the DNA will be sufficient to provide a therapeutic dose.

Actual delivery of the gene sequence, formulated as described above, can be carried out by a variety of techniques including direct injection, administration to the lung and other epithelial

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surfaces, intravenous injection and other physical methods.

The present invention also contemplates the use of TTP, and TNF $\alpha$  production (processing or secretion)-inhibitor polypeptide fragments thereof, as a pharmaceutical agent to effect suppression of TNF $\alpha$  biosynthesis, processing or secretion. Polypeptides can be made using commonly used and widely available techniques for the synthesis of synthetic peptides. The TTP protein or polypeptide fragments thereof can be synthesized recombinantly using common expression systems such as *E. coli*, baculovirus, Cos cells, etc. The protein/polypeptide can then be purified and used, for example, for injection or infusion as with many protein drugs currently available for clinical use. Alternatively, TTP can be isolated from natural sources, using art recognized techniques.

The TTP protein, or fragment thereof, can be administered by any appropriate means to achieve the effect sought (eg treatment of Type I diabetes, systemic lupus erythematosus, rheumatoid arthritis or other inflammatory condition, tumor, infection, or the like). Parenteral administration is preferred, for example, periodic subcutaneous, intramuscular, intravenous, intraperitoneal or intranasal routes can be used using either bolus injection or gradual infusion. Alternatively, topical or oral administration can be used.

The optimum dosage administered will vary with the subject, the protein/polypeptide and the effect sought. Appropriate doses can be readily determined by one skilled in the art.

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Compositions suitable for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions or emulsions. Compositions can be present in other dosage unit form (eg tablet or capsule). Compositions suitable for topical administration can be in the form of a cream, gel, ointment, lotion or foam.

The compositions comprise the TTP protein/polypeptide, in an amount effective to achieve the desired result, together with a pharmaceutically acceptable carrier.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow.

## EXAMPLES

The following experimental details pertain to the Examples I-V which follow.

Generation of TTP-deficient mice. A TTP insertion targeting vector was created by first isolating a 3.8 kb ~~2fp-35~~ (TTP genomic) clone from a SV129 library (Stratagene, La Jolla, CA) using a mouse TTP cDNA probe (Lai et al, J. Biol. Chem. 265:16556 (1990)); this fragment was cloned into the SalI site of BS+ (Stratagene). A 1.14 kb XhoI-BamHI ~~neo~~ fragment from pMC1PolA (Stratagene) was then ligated into the TTP SstI site (1 kb downstream of the initiator ATG) in pBS+/TTP. Next, a 4.9 kb SalI TTP-~~neo~~ fragment from pBS+/TTP-~~neo~~ was cloned into the SalI site of pSP73 (Promega, Madison, WI), into which two thymidine kinase genes (ClaI-BamHI and HindIII-XhoI fragments of pIC19R/MC1-TK) (Mansour et al, Nature 336:348 (1988)) had been cloned previously

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at the pSP73 ClaI-BamHI and HindIII-XhoI sites. This targeting vector was linearized with HindIII and electroporated into ES cells, which were then used to generate chimeric mice according to established methods (Koller et al, Proc. Natl. Acad. Sci. USA 86:8932 (1989)).

Northern blot analysis. Dissected tissues were rapidly frozen in liquid nitrogen, pulverized in liquid nitrogen, and then homogenized in a guanidinium thiocyanate solution, as described previously (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)). Total cellular RNA was isolated from the tissue lysate using an established acidic phenol extraction procedure (Chomzynski and Sacchi, Anal. Bioch. 162:156 (1987)). 15 µg RNA samples were separated by electrophoresis in 1.2% agarose/formaldehyde gels and used for northern blotting (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)) with a [<sup>32</sup>P]-labeled mouse cDNA probe (Lai et al, J. Biol. Chem. 265:16556 (1990)).

Cell culture and immunoprecipitation. Primary embryonic fibroblasts were prepared (Robertson, Robertson, E.J., ed. (IRL Press, Oxford) pp. 77-78 (1987)) from 14-17 day mouse embryos that had been generated from TTP(+/-) mouse matings. To identify (+/-) and (-/-) cell lines, DNA was isolated from the cells (Koller et al, Proc. Natl. Acad. Sci. USA 86:8932 (1989)), digested with EcoRI and subjected to Southern blot analysis (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)) using as a probe a 2.4 kb BstEII-HindIII TTP gene fragment (Taylor et al, Nucl. Acids Res. 19:3454 (1991)). Using this strategy, the 7.5 kb EcoRI fragment that resulted from a targeted TTP allele was easily distinguishable from the 10 kb EcoRI fragment that resulted from a wild-type TTP allele.

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Cell labeling and immunoprecipitation protocols have been described (Taylor et al, J. Biol. Chem. 270:13341 (1995). Briefly, confluent 60 mm plates of cells were serum-deprived for 24 h in DMEM supplemented with 1% (w/v) bovine serum albumin, and then exposed to [<sup>35</sup>S]-cysteine for 2 h and 20% (v/v) fetal calf serum for an additional 2 h. Next, the cells were lysed by brief sonication in a buffer containing 1% (w/v) nonidet P-40, 5mM EDTA, 0.15M NaCl, and 50mM Tris, pH8.3; protein was precipitated with an immunopurified polyclonal antiserum that recognized the 24 amino-terminal amino acids of TTF (Taylor et al, J. Biol. Chem. 270:13341 (1995)). Precipitated proteins were separated on 9% or 20% polyacrylamide SDS gels, which were dried and used for autoradiography.

Myeloid progenitor cell assays. Assays were performed on femoral bone marrow, peripheral blood and spleen from +/+ and -/- mice at 33 days of age (young mice) or 6.5 to 12 months of age (adult mice). These were performed as described (Cooper et al, Exp. Hematol. 22:186 (1994)). Marrow, spleen and blood cells were respectively plated at concentrations of  $2.5 \times 10^4$ ,  $2.5 \times 10^5$  and  $1.0 \times 10^5$  cells/ml in 1.0% methylcellulose culture medium with 30% fetal bovine serum (Hyclone, Logan, UT), 0.1 mM hemin, 1 U/ml recombinant (r) human (hu) erythropoietin (Epo, Amgen Corp., Thousand Oaks, CA), 5% vol/vol pokeweed mitogen mouse spleen cell conditioned medium (PWMSCM), and 50 ng/ml r murine (mu) steel factor (SLF; Immunex Corporation, Seattle, WA). Colonies were scored after 7 days incubation at 5% CO<sub>2</sub> and lowered (5%) O<sub>2</sub>. Calculation of the absolute numbers of progenitors per organ was based on the nucleated cellularity and colony counts for CFU-GM, BFU-E and CFU-GEMM in each organ for each individually assessed

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mouse. Cultures were also set up in methylcellulose or 0.3% agar (10% fetal bovine serum) in the presence or absence of different concentrations of Epo, rmu granulocyte-macrophage colony stimulating factor, rhu granulocyte colony stimulating factor, rhu macrophage colony stimulating factor (Immunex Corp) or PWMSCM with or without rmu SLF or rhu Flt-3 ligand (Immunex Corp) to assess the sensitivity of cells to stimulation by single or multiple cytokines.

Histological analysis. Mouse tissues were immersed in Bouin's fixative for 2 to 4 days, and then washed for several days in 70% (v/v) ethanol at room temperature. When required, tissues were decalcified following Bouin's fixation by immersing in 12.5% (w/v) sodium citrate and 25% (v/v) formic acid for 24 h, rinsing in running water for 24 h, and then washing for several days in 70% ethanol, all at room temperature. Fixed tissues were then embedded for paraffin sectioning; 5-7  $\mu$ m sections were stained with hematoxylin and eosin by standard methods, then photographed with a Nikon Opiphot-2 photomicroscope and Kodak Ektar 100 film.

Renal pathology was evaluated at 5 months of age. One kidney was fixed, embedded in paraffin and sectioned as described above prior to staining with hematoxylin and eosin, Congo red and periodic acid Schiff (PAS) (Tse, Mishell and Shiigi, eds. (W.H. Freeman and Company, New York), pp. 201-205 (1980)). The other kidney was quick frozen in OCT embedding compound on dry ice, and frozen sections were prepared for immunofluorescent microscopy using fluorescein-conjugated goat anti-mouse IgG or IgM as described (Andrews et al, Vet. Pathol. 31:293 (1994)).

Glomerular disease was graded by a pathologist blinded as to the genotype of origin of the kidney

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sections. Scores were determined using a grading system that assigns 0-3+ scores for proliferation, necrosis, crescent formation, vasculitis, and inflammatory infiltrate. IgG and IgM deposition were graded 0-3+ on the fluorescent slides by the same pathologist.

Fluorescent activated cell sorter (FACS)

analysis. Peripheral blood cells were obtained by capillary tube bleeding from the eye orbit; bone marrow cells were obtained by flushing dissected femurs with 2mL ice cold RPMI 1640 medium followed by gentle pipeting to disperse the cells; and splenocytes and thymocytes were obtained by macerating dissected tissues with the plunger of a disposable 1 ml syringe in ice-cold RPMI 1640 medium, and then isolating the cells by density centrifugation (Tse, Mishell and Shiigi, eds. (W.H. Freeman and Company, New York), pp. 201-205 (1980)). Some peripheral blood and bone marrow cells were stained with ACCUSTAIN Wright Stain (3WS10) as described by the manufacturer.

Analysis of cell surface phenotype was performed on the cell preparations according to previously described direct and indirect-immunofluorescence assays (Haynes et al, New Engl. J. Med. 304:1319-1323 (1981)), using a FACStar Plus Flow Cytometer and associated software (Becton Dickinson). The following directly conjugated monoclonal antibodies were used at saturating titers: Thy1.2 (anti-Thy-1, Becton Dickinson, Mountain View, CA), Ly-5 (anti-B220, Caltag, South San Francisco, CA) Lyt2 (anti-CD8, Becton Dickinson), L3T4 (anti-CD4, Becton Dickinson), OX-12 (anti-rat, Sera-Labs, Crawley Down, Sussex, England), and streptavidin-phycoerythrin (Pharmingen, San Diego, CA). PK-136 (American Type Culture Collection (ATCC) HB191) was purified from

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serum-free media (Gibco, Grand Island, NY) hybridoma culture supernatant using affinity chromatography over a Staphylococcal protein (A/G) column (Pierce, Rockford, NY), then fluorescein conjugated and used at saturating titer. The following hybridomas were cultured in serum-free medium and supernatant was used in indirect immunofluorescence flow cytometry assays using fluorescein-conjugated OX-12 as a secondary reagent: F4/80 (ATCC HB198), 14.8 (ATCC TIB164), Gr-1 (RB6-8CA, a gift of R.L. Coffman, DNAX, Palo Alto, CA), Ter119 (a gift of I.L. Weissman, Stanford University), and Y3-Ag1.2.3 (ATCC CRL 1631).

Evaluation of autoimmunity. DNA from calf thymus was purchases from the Sigma Chemical Co. DNA was dissolved in SSC (0.15M Na citrate, pH8) prior to purification by phenol extraction. Double-stranded DNA (dsDNA) was obtained by treating the DNA with S<sub>1</sub> nuclease while single-stranded DNA (ssDNA) was obtained by boiling for 10 min prior to rapid immersion in ice.

Sera obtained as described above were tested for reactivity to DNA antigens by ELISA as previously described (Gilkeson et al, J. Immunol. 151:1343 (1993)). Briefly, 96 well polystyrene plates were coated with DNA diluted to 5µg/ml in SSC. Antigens used in these assays were calf thymus dsDNA (dsDNA) and calf thymus single stranded DNA (ssDNA). After addition of DNA, plates were incubated for 2h at 37°C for ssDNA assays and 16 h at 37°C for dsDNA assays. Two-fold serial dilutions of sera in PBS-T (phosphate buffered saline containing 0.05% Tween 20) were then added to the plates starting at a 1/100 dilution. Following incubation, peroxidase conjugated goat-anti-mouse IgG was added. 3,3', 5,5' Tetramethylbenzidine (TMB) in 0.1M citrate (pH4) with



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0.015%  $H_2O_2$  was added for color development.  $OD_{380}$  absorbance was determined by a microtiter plate reader (Molecular Dynamics, Menlo Park, CA).

Rheumatoid factor (RF) activity in the sera was also determined by ELISA. For IgM RF assays, microtiter plates were coated with mouse IgG (Sigma) at 1  $\mu$ g/ml in PBS; for IgG RF assays, plates were coated with 1  $\mu$ g/ml of rabbit IgG in PBS. After blocking with 1% BSA in PBS, sera were added in dilutions beginning at a 1/100 dilution. Peroxidase conjugated goat anti-mouse IgG ( $\gamma$  chain specific) or goat anti-mouse IgM ( $\mu$  chain specific) were added to the plates followed by the substrate. After color development, absorbance at  $OD_{380}$  was determined.

*Crithidia luciliae* assays for anti-dsDNA were performed as suggested by the manufacturer (Kallestad, Austin, TX). Sera were tested at 1/20 and 1/50 dilutions.

Antinuclear antibody assays were performed as suggested by the manufacturer (Zeuss Scientific, Raritan, NJ). Sera were tested at 1/20 and 1/50 dilutions.

*TNF $\alpha$  antibody administration.* To test the possible role of  $TNF\alpha$  in the development of the TTP-deficient phenotype, six -/- mice received weekly intraperitoneal injections of a hamster monoclonal antibody (TN3-19.12) that is specific for mouse  $TNF\alpha$  (Sheehan et al, J. Immunol. 142:3884 (1989)); a general gift from Dr. Robert D. Schreiber, Washington University School of Medicine) and six -/- mice received an equivalent volume of PBS. The first injection occurred when the animals were 10 days of age, and continued at weekly intervals for a total of nine injections. The first two injections were 125  $\mu$ g of antibody in 50  $\mu$ l PBS, and the last seven injections were 250  $\mu$ g of antibody in 100  $\mu$ l of PBS.

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One week after the final injection, the animals were killed with CO<sub>2</sub>, and blood and tissues were harvested for blood counts and histology as described above.

## EXAMPLE I

## 5                      Generation of TTP (-/-) Mice

A targeting vector was constructed that contained 3.8kb of the gene encoding TTP, Zfp-36 (Taylor et al, Nucl. Acids Res. 19:3454 (1991)), in which a neomycin resistance gene (neo) was inserted into the protein-coding portion of the second exon. Insertion of this sequence introduced multiple stop codons upstream of the sequences encoding the two putative zinc fingers, precluding synthesis of functional TTP protein. Using this targeting vector and established experimental methods (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932 (1989)), TTP-deficient mice were generated. Among the first 492 offspring of heterozygous (+/-) crosses, 126 (26%) were homozygous wild-type (+/+), 267 (54%) were heterozygous (+/-), and 99 (20%) were homozygous null (-/-), indicating that there was no substantial embryonic lethality associated with the (-/-) genotype.

Northern analysis of tissues from (+/-) mice revealed that the endogenous TTP mRNA signal was decreased by about 50%, and that a TTP/neo fusion mRNA had been generated (Fig. 1a). No endogenous TTP mRNA was detected in tissues from a (-/-) mouse, but the TTP/neo fusion mRNA signal was increased over that seen in the +/- mice. Because the neo portion of the TTP/neo fusion mRNA contains many termination codons, a complete TTP/neo translation product should not be made; however, translation of an amino-

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terminal TTP fragment could have occurred. To test this possibility, primary embryonic fibroblasts were generated from both (+/-) and (-/-) embryos, and immunoprecipitations were performed on lysates from these cells, using an antibody (Taylor et al, J. Biol. Chem. 270:13341 (1995)) directed at the amino-terminus of TTP (Fig. 1b). Although TTP was readily detectable in (+/-) cells, neither intact TTP nor a truncated amino-terminal TTP fragment could be detected in the (-/-) cells (Fig. 1b).

The expression of two other mRNAs that encode related CCCH zinc finger proteins was also measured to determine if their expression was compensatorily increases in TTP-deficient mice; however, no change in expression of either TIS11B (cMG1) (Varnum et al, Oncogene 4:119 (1989), Taylor et al, Nucl. Acids Res. 19:3454 (1991)) or TIS11D (Varnum et al, Mol. Cell Biol. 11:1754 (1991)) was noted.

## EXAMPLE II

### Histological Characteristics of TTP (-/-) Mice

The (-/-) mice appeared normal at birth, but their rate of weight gain began to decrease compared to littermates between one and eight weeks after birth (Fig. 2). This failure of weight gain and eventual cachexia was one of the most striking characteristics of the phenotype, and occurred in essentially all of the mice to varying degrees (Fig. 2). They also developed patchy alopecia, dermatitis, arthritis, and conjunctivitis. Although all (-/-) mice eventually developed the syndrome, the degree to which they were affected was variable. 34% (of 56) were severely affected and died before reaching seven months of age. The remaining (-/-)

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mice were less severely affected, but nevertheless, 68% (of 37) displayed dermatitis, 88% arthritis, and 72% conjunctivitis by seven months of age. Survival of a (-/-) mouse was 16 months or more.

5       Histological examination of the (-/-) mice demonstrated several characteristic abnormalities. In the calvarial skin of seven-month old littermate mice, there was orthokeratotic hyperkeratosis in many areas, with an infiltration of neutrophils in the  
10       epidermis, and marked acanthosis. There was also diffuse inflammation in the underlying dermis, characterized by accumulation of many neutrophils and fewer lymphocytes, plasma cells, and macrophages. The inflammatory infiltrates extended to the deep  
15       dermal margins of the tissue, and in some cases, to the underlying skeletal muscle. Large numbers of Gr-1<sup>+</sup> neutrophils were present in both the epidermis and the dermis, while small foci of CD3<sup>+</sup>, TCR $\alpha$ <sup>+</sup>, and Thyl.2<sup>+</sup> lymphocytes, 75% of which were of the CD4<sup>+</sup>  
20       subset, were also present in the dermis. A striking finding was that subcutaneous fat was essentially absent as was mesenteric and epididymal fat.

      The epidermis of the eyelid was also thickened, was the palpebral conjunctiva. Neutrophils  
25       infiltrated the dermis beneath both the eyelid and the conjunctiva. In addition, there were relatively few mucous cells along the surface of the conjunctiva in the -/- mice.

      In most joints in both the front and rear paws  
30       of the (-/-) mice at seven months of age, the synovium was markedly inflamed and thickened, with proliferating synovial cells extending well into the joint spaces; the synovium contained many Mac-1<sup>+</sup> macrophages and fewer Gr-1<sup>+</sup> neutrophils, CD3<sup>+</sup>, TCR $\alpha$ <sup>+</sup>,  
35       and Thyl.2<sup>+</sup> lymphocytes and plasma cells. In addition, there was apparent proliferation of

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synovial cells and pannus formation, which in some cases completely separated apposing joint surfaces. Erosion of articular cartilage by pannus and extensive bone destruction were common. The marrow cavities were densely filled with cells of the myeloid lineage, especially mature neutrophils, and there was marked osteolysis of the inner aspect of the cortical bones.

Several abnormalities were also noted in the hematopoietic systems of the (-/-) mice. Thymuses in adult (-/-) mice were hypoplastic and showed no cortical/medullary organization; the thymuses of four days post-pactum (-/-) mice were decreased in size by an average of 50%. Spleens of the (-/-) mice were enlarged by an average of 41%, and there was extensive splenic myeloid hyperplasia, with many metamyelocyte, bands, and segmented neutrophils present. The perirenal, submaxillary, and mesenteric lymph nodes were also often enlarged, again showing extensive extramedullary hematopoiesis, primarily granulopoiesis. There was a marked increase in the number of myeloid cells in the bone marrow, which appeared nearly white in contrast to the red marrow of the control animals. Essentially all of the marrow myeloid cells were strongly Gr-1<sup>+</sup>. Although the cellular architecture of the liver appeared normal in (-/-) mice, foci of necrotizing hepatitis were present that contained a mixed inflammatory exudate of neutrophils, macrophages, and lymphocytes. There was also an inflammatory abscess in the interventricular septum of one mouse.

Because antinuclear antibodies were present (see below), the kidneys were examined histologically by staining with hematoxylin and eosin, periodic acid Schiff (PAS), and Congo red, and immunologically, for the presence and absence of IgG and IgM. The tubular

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and interstitial architecture was essentially normal in the kidneys from the -/- mice, but the glomeruli manifested increased cellularity and increased PAS-positive mesangial matrix. There was also focal, segmental thickening of peripheral capillary loops, which were congested with erythrocytes. IgG and IgM staining of glomeruli were similar in the kidneys from the +/+ and -/- mice. Proteinuria was not increased in the -/- mice compared to control, nor were plasma BUN and creatine significantly elevated compared to control.

## EXAMPLE III

## Hematopoietic Cell Populations in TTP (-/-) Mice

To further characterize the hematopoietic abnormalities seen in the (-/-) mice, complete blood counts and flow cytometric analyses of leukocyte subsets were performed (Fig. 3). In the (-/-) mice, the total peripheral white blood cell count was elevated by more than two fold [ $10.5 \pm 1.3$  (SEM)  $\times 10^3$  (n = 7) per  $\text{mm}^3$  vs.  $5.0 \pm 0.8 \times 10^3$  (n = 6) per  $\text{mm}^3$ ]. There was a marked increase in myeloid cells, with sharp increases in the number of Gr-1<sup>+</sup> neutrophils and F4/80<sup>+</sup> macrophages in peripheral blood and spleen, and in the number of Gr-1<sup>+</sup> neutrophils in bone marrow. The marrow myeloid cells were karyotypically normal, suggesting that they had not undergone malignant transformation. There were also increases in the number of PK136 natural killer cells in both peripheral blood and spleen. Conversely, there were smaller, less consistent decreases in B and T (B220) lymphocyte percentages and absolute numbers in hematopoietic tissues. Ly-5<sup>+</sup> B lymphocytes were decreased in peripheral blood, but were normal in

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spleen. The number of Thy-1' T lymphocytes in peripheral blood and spleen was normal, but the number of Thy-1' cells in bone marrow was decreased by two-fold. The peripheral red blood cell count, hemoglobin, hematocrit, and platelet count were within normal ranges; however, the Ter119' erythroid cells in bone marrow were decreased in percentage by about two-fold, presumably secondary to the massive increase of myeloid cells in the bone marrow.

- Assays of hematopoietic progenitor cells per organ were performed on cells from spleen, bone marrow and peripheral blood of young (age 33 days) and older (6.5 to 12 months) mice. In the young mice, absolute numbers of granulocyte-macrophage progenitors (CFU-GM) from -/- mouse bone marrow were increased approximately two-fold compared to control (+/+ (n=3):  $30.8 \pm 3.6 \times 10^3$ /femur (mean  $\pm$  SEM); -/-(n=3):  $64.3 \pm 0.5 \times 10^3$ /femur,  $p < 0.0025$  using Student's t test) whereas CFU-GM from spleen and peripheral blood were unchanged. Erythroid (BFU-E) and multipotential (CFU-GEMM) progenitors per spleen, femur and ml of peripheral blood were not significantly different ( $p > 0.05$ ) in the young -/- and +/+ mice. In the older mice, there were marked increases in myeloid progenitors (CFU-GM, BFU-E and CFU-GEMM) in spleen and peripheral blood but not bone marrow from the -/- compared to +/+ mice. Comparative progenitor cell values  $\times 10^3$  per spleen (-/- vs. +/+) were  $145 \pm 50$  vs.  $6 \pm 5$  for CFU-GM,  $115 \pm 51$  vs.  $7 \pm 4$  for BFU-E and  $10 \pm 4$  vs.  $0.2 \pm 2$  for CFU-GEMM. Comparative values per ml of blood were  $1530 \pm 781$  vs.  $46 \pm 39$  for CFR-GM,  $417 \pm 273$  vs.  $30 \pm 27$  for BFU-E and  $62 \pm 32$  vs.  $4 \pm 4$  for CFU-GEMM. Colonies from young or old -/- or +/+ mice did not form in vitro without addition of growth factors and no obvious differences in sensitivity of progenitor

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cells to stimulation of proliferation by single or multiple cytokines was apparent with bone marrow cells from -/- vs. +/+ mice.

5       Routine serum chemistries, including glucose, were within normal limits in the (-/-) mice, except for slightly decreased albumin and increases total serum globulin and  $\beta$ -globulin levels.

#### EXAMPLE IV

##### Autoantibodies

10       Rheumatoid factors (both IgG and IgM) and anti-Sm antibody titers were repeatedly normal in sera from the -/- mice. However, 3/4 of the sera from the -/- mice expressed high titers of antinuclear antibodies, with a homogenous pattern;  
15       these antibodies were not detected in 4/4 sera from the +/+ animals. The -/- sera (but not the +/+ sera) also contained antibodies to double-stranded DNA (mean +/- SEM of ELISA units from four -/- mice was 0.41 +/- 0.18 compared to 0.06 +/- 0.02 from four +/+  
20       mice;  $p = 0.11$  by Student's  $t$  test). This finding was confirmed by the Crithidia assay (4/4 -/- mice were positive, compared to 0/4 +/+ mice). Finally, sera from the -/- mice (but not from the +/+ mice) contained high titers of antibodies to single  
25       stranded DNA (mean +/- SEM ELISA units from four -/- mice was 1.49 +/- 0.30 compared to 0.22 +/- 0.05 from four +/+ mice;  $p = 0.006$  by Student's  $t$  test).



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## EXAMPLE V

TNF $\alpha$  Antibody Treatment

Because the phenotype of the TTP-deficient mice resembled that produced by chronic administration of TNF $\alpha$  (Keffer et al, EMBO J. 10:4025 (1991); Ulich et al, Res. Immunol. 144:347 (1993)), an attempt was made to prevent the development of the phenotype by treating the mice beginning at 10 days of age with nine weekly intraperitoneal injections of a hamster monoclonal antibody (TN3-19.12) that is specific for mouse TNF $\alpha$  (Sheehan et al, J. Immunol. 142:3884 (1989)). This antibody was originally thought to cross-react with TNF $\beta$  (Sheehan et al, J. Immunol. 142:3884 (1989)); however, subsequent work has shown that it does not neutralize the biological activity of this cytokine (R.D. Schreiber, personal communication). Four of six TTP -/- mice injected with PBS exhibited striking growth retardation, and one exhibited mild growth retardation (Fig. 4A). The sixth PBS-injected mouse died before growth retardation could become obvious (Fig. 4B); two others died before completion of the trial (Fig. 4). Three PBS-injected mice survived the trial, but were strikingly smaller than their wild-type littermates (Fig. 4). In contrast, five of six mice injected with the TNF $\alpha$  antibody maintained essentially identical growth curves to those of their +/+ and +/- littermates (Fig. 4), whereas one exhibited slight growth retardation (Fig. 8B). The mean body weights of the six TTP -/- mice receiving the TNF $\alpha$  antibody were in the middle of the normal range throughout the trial, whereas the mean weights of those receiving PBS were significantly ( $p < 0.001$ ) lower than those

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receiving antibody at all time points after week 4 (Fig. 4H).

The antibody-injected mice also developed none of the cutaneous or joint stigmata associated with the -/- syndrome. In addition, the -/- mice injected with TNF $\alpha$  antibodies did not display medullary myeloid hyperplasia, with marrow from these animals containing  $33.7 \pm 3.1\%$  (SEM) mature granulocytes and bands, compared to  $30.4 \pm 3.0\%$  for the wild-type animals ( $p = .49$ ). Every examined aspect of the TTP-deficient phenotype has been essentially normalized by the injection of TNF $\alpha$  antibody.

#### EXAMPLE VI

Bone Marrow Transplantation Reproduces  
TTP-Deficiency Syndrome in RAG-2 (-/-) Mice

##### Experimental details:

Mice: TTP (-/-) mice were generated as previously described (Taylor et al, Immunity 4:445 (1996)). Genotyping of offspring was performed by Southern blot analysis of tail DNA as described (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)), using as a probe an Sst-II/Sst-I 1.1 kb fragment of the genomic DNA; this contained 79 bp of the promoter, the entire first exon, the entire intron and 275 bp of the second exon (Lai et al, J. Biol. Chem. 270:25266 (1995)). RAG-2 (-/-) mice were obtained from a colony maintained at the Division of Laboratory Animal Resources, Duke University Medical Center, Durham, NC. All mice were maintained in autoclaved microisolator cages in a barrier facility, and fed with autoclaved food and acidified water.

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*Bone marrow transplantation:* Bone marrow was obtained from two male TTP (-/-) mice and their two male TTP (+/+) littermates at age six months. Bone marrow transplantation was performed as described (Linton et al, Science 267:1034 (1995)). Briefly, the mice were euthanized by CO<sub>2</sub> inhalation, and both femurs were aseptically removed. Bone marrow was flushed from the femurs with RPMI medium (GIBCO-BRL, Grand Island, NY) supplemented with 2% (v/v) FCS, 100 U/ml of penicillin, 100 µg/ml of streptomycin (P/S) (GIBCO-BRL) and 5 U/ml of heparin (Elkins-Sinn Inc., Cherry Hill, NJ). 10<sup>7</sup> total bone marrow cells in a final volume of 300 µl were injected intravenously through the tail vein into 10 RAG-2 (-/-) female mice (7-9 weeks old), 5 receiving marrow from TTP (-/-) mice [(-/-) recipient group] and 5 receiving marrow from TTP (+/+) mice [(+/+) recipient group]. Bone marrow cells from different donors were not pooled. Thus, two groups were established, one of 6 mice receiving the marrow from one pair of (-/-) and (+/+) mice, and another of 4 mice receiving the marrow from the other pair of mice. Mouse body weight was assessed weekly after the transplantation, and blood smears (tail bleeding) were performed monthly. One mouse each from the (-/-) and (+/+) recipient groups was analyzed 10 weeks after the transplantation. The remaining animals were analyzed at later time points, usually when the recipients of the (-/-) marrow were near death. Animals were euthanized by CO<sub>2</sub> inhalation, and careful autopsies were performed. When possible, blood was collected into heparinized tubes for cell counts. Non-anticoagulated venous blood (vena cava) was collected for blood smears and for serum. Blood smears were stained using the modified Wright stain Diff Quick Stain Set (Baxter Healthcare Corporation, McGaw Park, ILL). Tissues

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were fixed for 48 hr in Bouin's fixative and extensively washed in 70% (v/v) ethanol. Fixed tissues were then embedded in paraffin and 5  $\mu$ m sections were cut and stained with hematoxylin-eosin by standard techniques. Tissues containing bones were fixed in Bouin's for 48 hr, and then decalcified by immersion in 12.5% (w/v) sodium citrate/25% (v/v) formic acid for 24 hr, then washed in running water for 24 hr. They were then extensively washed in 70% ethanol and embedded in paraffin as for the soft tissues. Bone marrow was flushed from the femurs with RPMI/10% (v/v) FCS. Cells were deposited onto glass slides (cytopreps) using a cytocentrifuge (Shandon Inc., Pittsburgh, PA) and stained with the Diff Quick Stain Set. Imprints from spleen, lymph nodes and liver were prepared by sectioning the organ and briefly pressing the section onto a glass slide. Cells in these preparations were also stained with the Diff Quick Stain Set. Stained sections and cells were photographed using a Leitz Laborlux- 12 microscope (Ernst Leitz, Wetzlar GMBH, Germany) equipped with an Olympus PM-C35B camera (Olympus America Inc., Lake Success, NY).

*In situ hybridization histochemistry for presence of the Y chromosome:* Because marrow donors were male and recipients were female, it was possible to evaluate the presence of donor cells in the recipients by DNA in situ hybridization histochemistry using a Y chromosome probe. Tissue samples for this use were fixed for 24 hr in ice-cold ethanol/acetic acid (3:1) (v/v), and then extensively washed in cold 70% ethanol (v/v) before being embedded in paraffin. Bone marrow cytopreps were also fixed in ice-cold ethanol/acetic acid, and kept at -20°C until used. In situ hybridization

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histochemistry was performed using a mouse Y chromosome probe (Bishop et al, Nucl. Acids Res. 15:2959 (1987)) (provided by Dr. Colin E. Bishop (Baylor College of Medicine, Houston, TX)). The probe was labeled with digoxigenin with the DNA Labeling and Non-Radioactive Detection Kit (Boehringer Mannheim, Indianapolis, IN), following the instructions of the manufacturer. DNA in situ hybridization histochemistry was usually performed on bone marrow cells, but in selected cases, it was also performed on spleen, lymph node and liver sections. In situ hybridization was performed according to the protocol described by Keighren and West (Keighren et al, Histochem. J. 25:30 (1993)). The only modification was that bone marrow cytopreps were not treated with xylenes before rehydration in ethanol.

*Detection of autoantibodies:* Detection of anti-DNA and anti-nuclear antibodies was performed exactly as described previously (Taylor et al, Immunity 4:445 (1996)).

*Culture of peritoneal macrophages:* In order to investigate possible cellular sources of excess TNF $\alpha$  in the TTP (-/-) mice, studies were performed in mice prepared as described (Taylor et al, Immunity 4:445 (1996)) that were not involved in the marrow transplantation studies. Peritoneal macrophages were prepared from two TTP (-/-) and their two TTP (+/+) litter-mate mice at 3 months of age. The mice were euthanized by CO<sub>2</sub> inhalation, and 5-10 ml of serum-free DMEM (GIBCO-BRL) were injected in the peritoneal cavity. The abdomen was gently massaged and a longitudinal incision was performed in the abdominal wall to allow recovery of the injected medium. The peritoneal cavity was then washed with an additional

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5-10 ml of medium, to obtain the greatest yield of macrophages. Recovered cells were counted on a hemocytometer. Purity of the macrophage population was assessed by Giemsa stain and nonspecific esterase activity (Tucker et al, J. Immunol. Methods 14:267 (1977)). Macrophages were plated at  $2.5 \times 10^5$  cells/well in 96-well plates, in DMEM supplemented with 10% (v/v) FCS and different concentrations of LPS (Sigma Chemical Co., St Louis, MO) (0, 0.1, 1, 10, 100 ng/ml), and cultured for 24 hr. The supernatants were then assayed for TNF $\alpha$  accumulation as described below.

Culture of fetal liver macrophages: Fetal liver macrophages were obtained from hematopoietic progenitors present in fetal liver. Pregnant TTP (+/-) females that had been mated with TTP (+/-) males were euthanized by CO<sub>2</sub> inhalation on day 14-16 of gestation and the fetuses were removed. Fetal livers were carefully and aseptically removed and single cell suspensions were obtained by dispersing the tissue between two glass slides in harvesting medium (GIBCO-BRL) supplemented with 10% (v/v) FCS, 15 mM HEPES (pH 7.4), 0.2% (w/v) sodium bicarbonate, P/S and 2 mM glutamine]. Fetal liver macrophages were prepared from these suspensions according to the method described by Warren and Vogel (Warren et al, J. Immunol. 134:982 (1985)) for bone marrow-derived macrophages, and cultured for two weeks in the presence of harvesting medium supplemented with 30% TKL cell (ATCC, Rockville, MD)-conditioned medium, prepared as described (Hume et al, J. Cell. Physiol. 117:189 (1983)). After two weeks in culture, cells were harvested with the neutral protease Dispase H (Boehringer Mannheim, Indianapolis, IN) [1.5 mg/ml in Ca<sup>2+</sup>/Mg<sup>2+</sup> free Earle's balanced salt solution], as

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described (Warren et al, J. Immunol. 134:982 (1985)), except that the cells were resuspended in RPMI supplemented with 2 mM glutamine, P/S, 30 mM HEPES (pH 7.4) and 0.4% (w/v) sodium bicarbonate, using 10% (v/v) instead of 2% FCS. Purity of the preparations was assessed by morphology (Diff Qick Stain Set) and non-specific esterase activity. By these criteria, the cells obtained by this method were more than 90% macrophages. Macrophages were then plated at  $1.25 \times 10^6$  cells/ml on 96-well plates and incubated in 200  $\mu$ l of the same medium at 37°C for 24 hr before being stimulated for another 24 hr with increasing concentrations of LPS (0, 1, 10, 100, 1000 ng/ml). In another series of studies, cells were plated in 60 mm dishes, and incubated in the presence or absence of  $\mu$ g/ml LPS for 4 hr. In all cases, supernatants were harvested and assayed for TNF $\alpha$  accumulation by western blot, as described below. Cells from the 4 hr LPS stimulation were harvested and used for the measurement of TNF $\alpha$  and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, as described below.

#### *Culture of bone marrow-derived macrophages:*

Bone marrow-derived macrophages were prepared as described by Warren and Vogel (Warren et al, J. Immunol. 134:982 (1985)). Bone marrow cells from both humeruses and both femurs were isolated from five TTP (-/-) and five TTP (+/+) mice (age 6-7 months). Cells were cultured as described above for fetal liver macrophages, except that at day 1, cells were split in two T75 CM<sup>2</sup> flasks (Becton Dickinson, Franklin Lakes, NJ), instead of one, as usually done for fetal liver macrophages, and the cells were cultured for only 10 days. At that point, they appeared to be essentially 100% macrophages, as

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determined by morphology and nonspecific esterase staining. On day 10, the cells were rinsed once with RPMI, 2 mM glutamine, P/S, 30 mM HEPES (pH 7.4), 0.04% (w/v) sodium bicarbonate and 10% FCS, and  
5 incubated in the same medium for 24 h. At that point, the medium was removed and replaced by fresh medium after one wash, and the cells were incubated in the presence or absence of 1 µg/ml LPS for 4 hr. The supernatants were then harvested and assayed for TNFα  
10 accumulation by western blot, as described below; the cells were harvested and used for the measurement of TNFα and glyceraldehyde-3-phosphate dehydroxogenase (GAPDH) mRNA levels, as described below.

*Preparation of B and T lymphocytes:* B and T  
15 lymphocytes were prepared from spleen and thymus of 11 day old TTP (+/+) and (-/-) littermate mice. The animals were euthanized by CO<sub>2</sub> inhalation, and spleen and thymus were aseptically removed. Tissues were placed in 10 ml of RPMI supplemented with 10% (v/v)  
20 FCS, P/S and 2 mM glutamine. Single cell suspensions from thymus were obtained according to the protocol described by Kruisbeek (Kruisbeek, A.M. In Current Protocols in Immunology., Coligan et al, editors. John Wiley and Sons, Inc., New York, NY (1995)). B  
25 lymphocytes were purified from spleens by complement mediated lysis of T cells, as described (Sato et al, Proc. Natl. Acad. Sci. USA 92:11558 (1995)), using the monoclonal antibodies H57-597 (anti-TCR) and 145-2C115 (anti-CD3) (Nishimura et al, Immunology 83:196  
30 (1994)) (provided by Dr. Thomas F. Tedder, Duke University, Durham, NC). B and T lymphocytes were cultured at 10<sup>6</sup> cells/ml in RPMI/10% FCS, in the presence of different agents [10 ng/ml of recombinant murine IL-2 (R&D Systems, Minneapolis, MN), 1 µg/ml  
35 PHA (Sigma Chemical Co.) and 1 µg/ml LPS for T



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lymphocytes; increasing concentrations of LPS for B lymphocytes (0, 1, 10, 100, 1000 ng/ml)] for 24 hr, and supernatants were harvested to measure the accumulation of TNF $\alpha$  by western blot, as described below.

Western blotting: Supernatants from cultured macrophages, and B and T lymphocytes, were removed, centrifuged at 15,000 rpm in an Eppendorf benchtop centrifuge for 10 min at 4°C to eliminate any floating cells, and mixed with 1/5 volume of 5X SDS-sample buffer (Blackshear, P.J., Methods Enzymol. 104:237 (1984)). Volumes of supernatant corresponding to  $1.25 \times 10^5$  cells were loaded onto 16% SDS-polyacrylamide gels (Protogel, National Diagnostics, Atlanta, GA), and subjected to electrophoresis at 40 V for 16 hr. Proteins were then transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) for 2 hr in 20% methanol, 150 mM glycine and 48 mM Tris-HCl (pH 8.3) (845 mAmp, room temperature), using a Hoeffer Transphor Electro-transfer Unit (Hoeffer, San Francisco, CA). After transfer of the proteins, the membranes were incubated in 7.5% (w/v) non-fat milk in Tris-buffered saline (TBS)/0.5% (v/v) Tween 20 at room temperature for 1 hr. The primary antibody against TNF $\alpha$ , a rabbit-anti-mouse antiserum (Kull et al, J. Cell. Biochem. 42:1 (1990)) (provided by Dr. Fred Kull (Glaxo Wellcome Inc., Research Triangle Park, NC) was diluted 1:2500 in TBS/0.5% Tween 20, and was incubated with the membranes for 1 hr at room temperature. The membranes were then washed in TBS/0.5% Tween 20 (2x5 min, 2x10 min) and then secondary antibody (goat-anti-rabbit, horseradish peroxidase-conjugated, Bio-Rad, Hercules, CA) was added at a 1:5000 dilution in the same buffer and

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incubated for a further 30 min at room temperature. The membranes were washed again four times as described above and developed with the Amersham ECL Detection System (Amersham Corporation, Arlington Heights, IL), as recommended by the manufacturer. After ECL, films were analyzed using a Zeineh Soft Laser Scanning Densitometer, model SL-504-XL (Biomed Instruments, Inc., Fullerton, CA).

*Northern blotting:* Total cellular RNA was obtained from marrow macrophage cultures and fetal liver-derived macrophages cultures using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH), according to the method described by Chomczynski (Chomczynski, P., BioTechniques 15:532 (1993)). Genomic DNA was isolated from the same samples and used to estimate the number of cells present in each sample. 10 µg of RNA for the bone marrow-derived macrophages and 3.8 µg from the fetal liver-derived macrophages were separated on 1.2% agarose/formaldehyde gels and processed for northern blotting as described (Stumpo et al, Proc. Natl. Acad. Sci. USA 86:4012 (1989)). The filters were successively probed with a mouse cDNA for TNFα (ATCC) and a rat cDNA for GAPDH (Tso et al, Nucl. Acid Res. 13:2485 (1985)). PhosphorImager analysis was used for quantitation of the blots; the results were expressed as quotients of TNFα mRNA ÷ GAPDH mRNA from each animal, and these quotients were then averaged and compared using Student's t test.

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Results

5 RAG-2 (-/-) female mice at 7-9 weeks of age received bone marrow from either TTP (-/-) or (+/+) male mice. None of the recipient mice showed any acute adverse effects within one month of transplantation that could suggest graft versus host disease. Engraftment was assessed by the appearance of mature lymphocytes in peripheral blood, which occurred within one month of transplantation in all 10 recipients of both TTP (-/-) or (+/+) bone marrow. At various times during the study, recipient animals were euthanized and analyzed pathologically; at each point, a (-/-) marrow recipient was compared to a (+/+) marrow recipient.

15 Body weight: Loss of weight or failure to gain weight normally is one of the most prominent aspects of the TTP-deficiency phenotype (Taylor et al, Immunity 4:445 (1996)). No evident differences in the growth curves of the (+/+) and (-/-) recipients were apparent for the first 3 months after transplantation. However, three of the five recipients of (-/-) marrow then exhibited varying degrees of weight loss, ranging from profound (mouse #8) to moderate (#10 and #2). Of the recipients of (-/-) marrow, mouse #1 was selected randomly for pathological evaluation at 10 weeks after transplantation, before any of the animals showed evidence of weight loss, and mouse #7 never exhibited weight loss, despite repeated detection of circulating lymphocytes. None of the five recipients of (+/+) marrow exhibited weight loss or clinical or pathological abnormalities.

The weight loss was accompanied by nearly complete absence of adipose tissue in any of the

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usual fat depots (subcutaneous, mesenteric, perirenal) in 3/5 of the mice receiving (-/-) marrow (see below). Even mouse #1, randomly selected for pathological examination 10 weeks after  
5 transplantation, exhibited less adipose tissue than its control. Mouse #7 was the only recipient of TTP (-/-) marrow that appeared to have normal amounts of fat.

*Histology:* Histological evaluation of the  
10 recipients of (-/-) marrow revealed evidence of both medullary and extramedullary myeloid hyperplasia, as described previously for the TTP-deficient mice (Taylor et al, Immunity 4:445 (1996)). The bone marrow from all of the (-/-) recipient mice appeared  
15 grossly white, in contrast to the red marrow from the (+/+) recipients; this difference was consistently observed in previous study of the TTP (-/-) and (+/+) mice (Taylor et al, Immunity 4:445 (1996)). Marrow from all of the (-/-) recipients exhibited an  
20 increase in myeloid cells compared to the (+/+) recipients [73.8  $\pm$  11.5% myeloid cells in the (-/-) recipients (n=5) versus 47.2  $\pm$  8% myeloid cells in the (+/+) recipients (mean  $\pm$  SD, n=5), p=0.007].

Peripheral blood white cell counts were  
25 increased only in mouse #2 [ $98 \times 10^3/\mu\text{l}$  compared to the control range of  $5.7 \pm 2.1 \times 10^3/\mu\text{l}$  (n=2)]. Peripheral blood smears from this mouse showed an increase in circulating myeloid cells, both from the granulocytic and monocytic lineages [77.3% myeloid  
30 cells (monocytes + granulocytes) in mouse #2, versus 52% myeloid cells in the age-matched controls].

Livers from the (-/-) recipient mice appeared grossly normal at autopsy in four out of five animals. However, mouse #2 exhibited significant  
35 enlargement of the liver [1.8 g, compared to  $1.2 \pm$

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0.3 g (n=2) in the age-matched controls]. Multiple white nodules were apparent on the surface of the organ; these consisted largely of granulocytes, which also infiltrated the rest of the hepatic parenchyma. Imprints from these nodules confirmed the presence of myeloid cells at different stages of differentiation. The liver from mouse #7 also contained foci of granulocytes, with massive granulocytic infiltration of the gall bladder wall. Abnormal foci of granulocytes were not detected in the livers of the other three (-/-) recipients, or in the livers of any of the (+/+) recipients.

Splenomegaly was not generally present in the (-/-) recipient mice, except for mouse #2, whose spleen weighed 374 mg (compared to  $90.5 \pm 2.8$  mg in age-matched controls, n=2). However, all (-/-) recipients exhibited infiltration of the spleen with myeloid cells. They also contained increased numbers of megakaryocytes compared to the (+/+) recipient spleens. Imprints of the spleens from the (-/-) recipients revealed myeloid cells in all stages of differentiation. In the (-/-) recipients, the increase in the number of myeloid cells was accompanied by destruction of the normal architecture of the spleen, without the clear distinction between the white and red pulp.

Mouse #2 [(-/-) recipient] also exhibited lymphadenopathy, in which myeloid cells were also present.

The skin from the (+/+) recipients appeared normal. In 3 out of 5 (-/-) recipient mice, the skin completely lacked subcutaneous fat, as seen in TTP (-/-) mice (Taylor et al, Immunity 4:445 (1996)). Some animals exhibited a perivascular inflammatory infiltrate in the dermis.

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The carpal joints from the (-/-) recipient mice showed varying extents of inflammatory pannus, bone erosion and bone destruction. As in the TTP-deficient mice (Taylor et al, Immunity 4:445 (1996)),  
5 there was generalized enlargement of the marrow cavities in all of the (-/-) recipients, which were full of active hematopoietic cells, particularly of the myeloid lineage. In contrast, the (+/+) recipients exhibited mostly fat and very few  
10 hematopoietic cells in the marrow. Neither mouse #1, which was analyzed only 10 weeks after marrow transplantation, nor mouse #7, which appeared to be healthy after 10 months of follow up, exhibited an inflammatory pannus in the carpal joints. However,  
15 mice #2, #8, and #10 exhibited varying amounts of inflammatory pannus that completely destroyed the bone surface and articular cartilage and infiltrated the marrow cavities.

**Autoantibodies:** Four months after marrow  
20 transplantation, only mouse #2 exhibited antinuclear antibodies and anti-DNA antibodies, both by ELISA and the Chrithidia assay. However, by the end of the study, all three (-/-) recipients studied were positive for antibodies to both single- and double--  
25 stranded DNA [for single-stranded DNA the mean  $\pm$  SD of ELISA units from the 3 (-/-) recipient mice was  $0.52 \pm 0.42$  compared with  $0.014 \pm 0.005$  for the two (+/+) recipients; for double-stranded DNA, the values were  $0.5 \pm 0.37$  for the (-/-) recipients versus  $0.034 \pm 0.026$  for the (+/+) recipients]. Anti-nuclear  
30 antibodies were positive in mouse #2, but negative in the other two (-/-) recipients and in both of the (+/+) recipients studied.

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*In situ* hybridization for Y chromosome: *In situ* hybridization with a DNA probe for mouse chromosome Y was performed in an attempt to establish the origin of the myeloid cells that were present in the transplanted animals. In bone marrow there was a mixed population of myeloid cells positive for the Y chromosome (from the donor) and cells that were negative (from the recipient) in both (-/-) and (+/+) marrow recipients. In mouse #2, *in situ* hybridization was also performed on sections of spleen, lymph nodes and liver. Virtually the entire spleen and lymph nodes showed an intense hybridization signal, mainly due to the presence of mature lymphocytes originating from the donor. In the liver, only granulocytes and myeloid cells were positive for the Y chromosome, whereas the hepatocytes (recipient) were completely negative. However, the number of Y chromosome positive granulocytes in the liver represented only about 50% of the total granulocytes present, as determined by hematoxylin-eosin staining.

*TNF $\alpha$  production:* Western blot studies were performed on supernatants from cultured macrophages and B and T lymphocytes in order to assess the production of TNF $\alpha$  by these cells. Three different sources of macrophages were used: resident peritoneal macrophages, obtained by lavage of the peritoneal cavity of adult mice; fetal liver-derived macrophages, obtained by culturing the hematopoietic precursors present in the fetal liver between days 14-16 of gestation; and bone marrow-derived macrophages, obtained by culturing the hematopoietic precursors from the bone marrow of adult mice.

In all three cases, macrophages obtained from TTP (-/-) animals showed an increased accumulation of

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TNF $\alpha$  in the medium when stimulated with LPS. Thus, peritoneal macrophages from TTP (-/-) mice, incubated for 24 hr in the presence of 10 ng/ml of LPS, secreted readily detectable amounts of TNF $\alpha$  into the culture medium. By contrast, under the same conditions, peritoneal macrophages from TTP (+/+) mice did not produce detectable amounts of TNF $\alpha$  under western blot conditions used. In these mice, measurable TNF $\alpha$  accumulation only occurred after 24 hr of incubation in the presence of 100 ng/ml LPS, and even then, the levels of TNF $\alpha$  were much lower than those observed for the macrophages from the TTP (-/-) mice. Densitometry of the autoradiographs indicated that there was a 5.6-fold increase in the levels of TNF $\alpha$  present in the supernatants from the TTP (-/-) peritoneal macrophages compared to the TTP (+/+) cells exposed to 100 ng/ml LPS.

Macrophages derived from fetal liver exhibited essentially the same behavior. TNF $\alpha$  was readily detectable in the culture supernatants after 24 hr in the presence of 1 ng/ml LPS, but at much higher levels in the (-/-) macrophages than in the (+/+) ones. These autoradiographs were analyzed by densitometry, and the results compared using Student's t test. At all LPS concentrations studied, there was about a five-fold greater accumulation of TNF $\alpha$  in the supernatants from the (-/-) cells compared to the (+/+) cells; these differences were statistically significant ( $p < 0.05$ ). Longer exposure of these blots showed that, even in the absence of LPS, there was greater TNF $\alpha$  accumulation in the supernatants from the cells compared to the (+/+) cells.

Bone marrow-derived macrophages were exposed to 1  $\mu$ g/ml LPS for 4 hr, and the levels of TNF $\alpha$  present in the culture supernatants were assayed by western



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blot. In all cases, the media from the (-/-) macrophages contained much higher levels of TNF $\alpha$  than the media from the cells. Densitometry of these autoradiographs revealed that there was a seven-fold greater accumulation of TNF $\alpha$  in the supernatants from the (-/-) cells compared to the (+/+) cells; this difference was statistically significant ( $p < 0.01$ ).

Analysis of TNF $\alpha$  production by B and T lymphocytes obtained from TTP (-/-) and mice did not reveal any significant differences between cells of the two genotypes. TNF $\alpha$  mRNA levels were studied in fetal liver-derived macrophages and in bone marrow-derived macrophages. In bone marrow-derived macrophages, the basal levels of TNF $\alpha$  mRNA were similar in (-/-) and (+/+) cells when normalized for GAPDH mRNA levels. However, after LPS stimulation (1  $\mu$ g/ml for 4 hr), the TNF $\alpha$  mRNA levels in (-/-) cells were approximately twice as great as the ones from the (+/+) cells ( $p < 0.05$ ). A similar two-fold difference was observed in the fetal liver-derived macrophages.

*Conclusions:* The above-described studies indicate that macrophage progenitors are among the transplanted cells that are capable of reconstituting the TTP-deficiency phenotype in RAG-2 (-/-) mice. These studies also indicate that macrophages are among the sources of TNF $\alpha$  overproduction in the TTP (-/-) animals.

\* \* \*

All documents cited above are hereby incorporated in their entirety by reference.

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One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

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WHAT IS CLAIMED IS:

1. A method of inhibiting tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) production, processing or secretion in a mammal comprising increasing the level of tristetraprolin (TTP), or a TNF $\alpha$  production, processing or secretion-inhibitory polypeptide fragment thereof, in said mammal so that said inhibition is effected.

2. The method according to claim 1 wherein said level of TTP is increased by administering to said mammal a nucleic acid sequence encoding TTP, or said polypeptide fragment thereof, and effecting expression of said sequence so that said level of TTP, or polypeptide fragment thereof, is increased.

3. The method according to claim 2 wherein said nucleic acid sequence encodes TTP.

4. The method according to claim 2 wherein said nucleic acid sequence is present as an insert in an expression vector.

5. The method according to claim 4 wherein said vector is a viral vector.

6. The method according to claim 2 wherein said nucleic acid sequence is present in a liposome.

7. A method of treating an effect of excess TNF $\alpha$  in a mammal comprising administering to said mammal TTP, or polypeptide fragment thereof that inhibits TNF $\alpha$  production, processing or secretion, or agent that enhances a TNF $\alpha$  production, processing or

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secretion-inhibitory effect of TTP, in an amount sufficient to effect treatment.

8. The method according to claim 7 wherein said TTP, or said polypeptide fragment thereof, is administered by introducing into said mammal a nucleic acid sequence encoding said TTP, or said polypeptide fragment thereof, and effecting expression of said sequence.

9. The method according to claim 7 wherein said effect of said excess is inflammation, infection or cancer cachexia.

10. The method according to claim 7 wherein said effect is rheumatoid arthritis, Type I diabetes or systemic lupus erythrematosis.

11. A method of identifying a subject susceptible to a TNF $\alpha$  associated disease or disorder comprising isolating a DNA-containing biological sample from said subject, locating the TTP gene present in said DNA and comparing the nucleic sequence of said TTP gene with a wild-type TTP encoding sequence and thereby determining whether said TTP gene includes a mutation that renders said subject susceptible to said disease or disorder.

12. The method according to claim 11 wherein, after locating the TTP gene, at least one polymorphic locus linked to said TTP gene is identified.

13. A method of identifying a subject susceptible to a TNF $\alpha$  associated disease or disorder comprising

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- i) isolating a biological sample from said subject,
- ii) contacting said sample with a TTP binding partner under conditions such that complexation between TTP and said binding partner can occur, and
- iii) detecting the presence or absence of said complexation, or comparing the extent of said complexation with a control sample comprising wild-type TTP.

14. The method according to claim 13 wherein said binding partner is an anti wild-type TTP antibody.

15. A method of screening a compound for its ability to enhance the ability of TTP to inhibit TNF $\alpha$  production comprising

- i) contacting said compound with a sample comprising a TNF $\alpha$  encoding sequence, in the presence of TTP or TNF $\alpha$  production-inhibitory polypeptide fragment thereof, under conditions such that said TNF $\alpha$  encoding sequence can be expressed, and
- ii) determining the level of expression of said TNF $\alpha$  encoding sequence and comparing that level to a level of expression obtained in the absence of said compound.

16. A method of screening a compound for its ability to enhance a TNF $\alpha$  transcription-repressor effect of TTP comprising

- i) contacting said compound with a sample comprising a TNF $\alpha$  promoter sequence operably linked to an encoding sequence, in the presence of TTP or TNF $\alpha$  production-inhibitory polypeptide fragment thereof, under conditions such that said encoding sequence can be expressed, and

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ii) comparing the level of expression of said encoding sequence obtained to a level of expression obtained in the absence of said compound.

17. The method according to claim 16 wherein said encoding sequence encodes a reporter protein.

18. The method according to claim 16 wherein said encoding sequence encodes TNF $\alpha$ .

19. A method of screening a compound for its ability to enhance a TNF $\alpha$  mRNA translation-inhibitory effect of TTP comprising

i) contacting said compound with a sample comprising TNF $\alpha$  mRNA, in the presence of TTP or a TNF $\alpha$  translation-inhibitory fragment thereof, under conditions such that translation of said TNF $\alpha$  mRNA can be effected, and

ii) determining the level of translation of said TNF $\alpha$  mRNA and comparing that level of translation of said TNF $\alpha$  mRNA to a level of translation of TNF $\alpha$  mRNA obtained in the absence of said compound.

20. A method of screening a compound for its ability to enhance the ability of TTP to inhibit TNF $\alpha$  processing comprising

i) contacting the compound with a sample comprising TNF $\alpha$  and TTP or TNF $\alpha$  processing-inhibitory polypeptide fragment thereof, and

ii) determining the level of processed TNF $\alpha$  in said sample and comparing that level to a level obtained in the absence of said compound.

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21. A method of screening a compound for its ability to enhance the ability of TTP to inhibit TNF $\alpha$  secretion from a cell comprising

i) contacting a cell comprising TNF $\alpha$  and TTP or a TNF $\alpha$  secretion-inhibitory polypeptide fragment thereof, with said compound, and

ii) determining the amount of TNF $\alpha$  secreted from said cell and comparing that amount to an amount obtained in the absence of said compound.

22. A TTP-deficient non-human mammal.

23. The mammal according to claim 22 wherein said mammal is a rodent.

24. The mammal according to claim 22 wherein a TTP gene present in the genome of said mammal is disrupted.

25. A method of screening or testing a compound for its ability to treat a symptom of excess TNF $\alpha$  comprising administering said compound to said mammal according to claim 22 and monitoring the effect of said compound on said symptom.

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KIDNEY LIVER LUNG SPLEEN THYMUS

+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-
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28S—

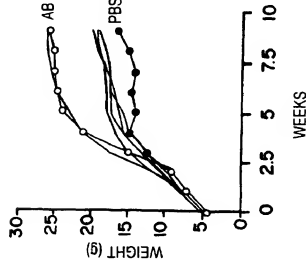
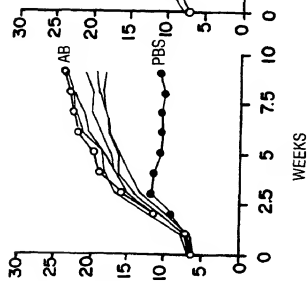
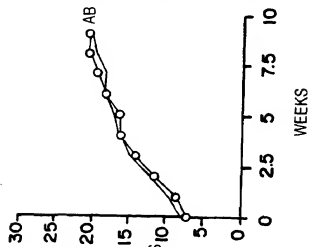
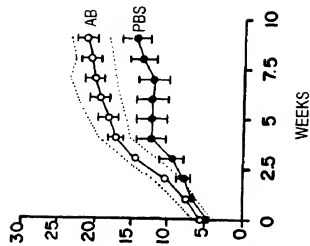
18S—

**Fig. 1A**

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**Fig. 4E****Fig. 4F****Fig. 4G****Fig. 4H**

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Fig. 4A

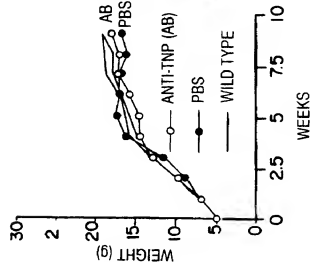


Fig. 4B

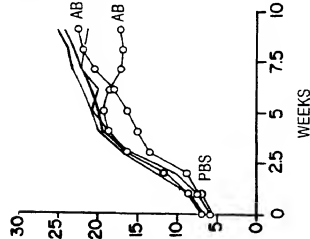


Fig. 4C

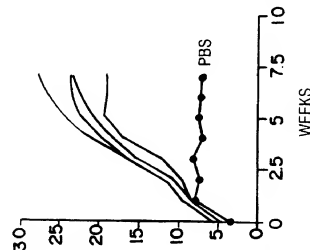
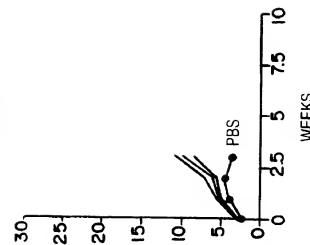
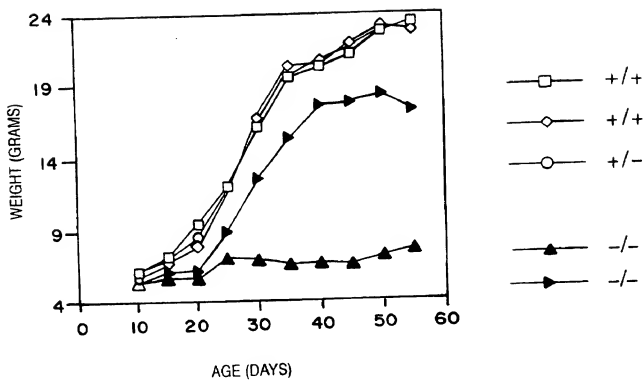


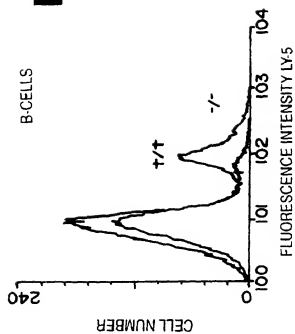
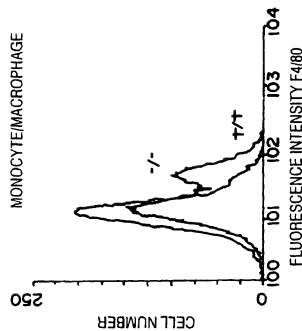
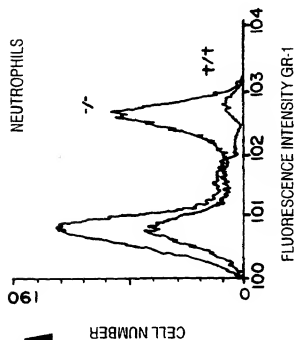
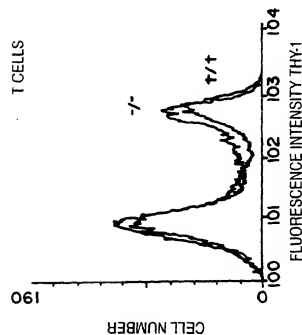
Fig. 4D



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**Fig. 2**

**Fig. 3B****Fig. 3D****Fig. 3A****Fig. 3C**

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/08394

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US 5,580,859 A (FELGNER et al.) 03 December 1996, see entire document.	1-6
Y,P	US 5,576,206 A (SCHLEGEL) 19 November 1996, see entire document.	1-6
Y,P	US 5,567,433 A (COLLINS) 22 October 1996, see entire document.	1, 2, 6-10
Y,P	US 5,547,970 A (WEITHMANN et al.) 20 August 1996, see entire document.	7-10, 15-21, 25
Y,P	US 5,519,000 A (HEAVNER et al.) 21 May 1996, see entire document.	7-10, 15-18, 25
Y	US 5,506,340 A (HEAVNER) 09 April 1996, see entire document.	7-10, 15-18, 25
Y	US 5,486,595 A (HEAVNER) 23 January 1996, see entire document.	7-10, 15-18, 25
Y	US 5,428,132 A (HIRSCH et al.) 27 June 1995, see entire document.	1-5, 7-10
Y	US 5,252,479 A (SRIVASTAVA) 12 October 1993, see entire document.	1-5, 7-10

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International application No.  
PCT/US97/08394

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 43/04; C12Q 1/68; G01N 33/566

US CL : 514/2, 44; 435/6; 436/501; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 44; 435/6, 235.1, 320.1; 436/501; 800/2; 514/2; 530/350; 536/23.1, 23.5, 24.31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,E	US 5,641,751 A (HEAVNER) 24 June 1997, see entire document.	7-10, 15-18, 25
Y,E	US 5,650,156 A (GRINSTAFF et al.) 22 July 1997, see entire document.	1-6
Y,E	US 5,648,251 A (KOTANI et al.) 15 July 1997, see entire document.	1-6
Y,E	US 5,646,154 A (IRIE et al.) 08 July 1997, see entire document.	7-10, 19-21
Y,E	US 5,641,680 A (ZHAO) 24 June 1997, see entire document.	1-6
Y,E	US 5,635,380 A (NAFTILAN et al.) 03 June 1997, see entire document.	1-6

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* "A" document defining the general state of the art which is not considered to be of particular relevance	* "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* "E" earlier document published on or after the international filing date	* "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combinations being obvious to a person skilled in the art
* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
* "O" documents referring to an oral disclosure, use, exhibition or other means	* "A" document member of the same patent family
* "P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

24 JULY 1997

Date of mailing of the international search report

29 AUG 1997

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.  
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**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

APS

Search Terms: tumor necrosis factor, triastetraprolin, TTP, gene therapy, viral vector, liposome, inhibit? expression, TTP-deficient, liposome, blackheart/in

